

RICE UNIVERSITY

Anticipation of Nitric Oxide Stress in the Human Commensal Fungus *Candida albicans*

by

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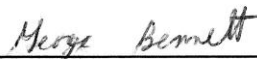
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ABSTRACT

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Candida albicans is the most common human commensal fungus, able to colonize host niches such as skin, mouth and gastrointestinal tract. Colonization of diverse microenvironments requires the ability to evade or overcome innate host protection and adapt to rapid transitions between environments with different stresses and nutrient availability. Colonization of the gastrointestinal tract requires passage through the stomach containing toxic levels of nitric oxide, generated from acidification of nitrite in the low pH of the stomach. Although resistance of *C. albicans* to nitric oxide is mediated by the flavohemoglobin Yhb1, little is known about the physiologically relevant ligands that regulate *YHB1* expression. Here I propose the hypothesis that nontoxic saliva chemicals induce *YHB1* expression and promote resistance to nitric oxide generated in the stomach.

Supporting this hypothesis is the observation that two ions actively concentrated in the saliva – nitrate and thiocyanate – induce *YHB1* expression. Indeed, whole-genome transcriptional analysis of *C. albicans* treated with nitrate or thiocyanate produce gene expression profiles nearly identical to cells treated with nitrite or nitric oxide.

Pretreatment of *C. albicans* with either of these two nontoxic compounds increases resistance of the yeast to nitric oxide. I propose that this is an evolved response in which *C. albicans* anticipates nitric oxide stress generated in the stomach. *C. albicans* thus upregulates nitric oxide stress response genes in response to saliva signals that precede

nitric oxide formation further on in the gut. Only a few examples of anticipatory signaling have so far been identified and it is not known how common this type of regulation is among microbes.

Expression of the *YHB1* gene in response to nitric oxide is regulated by the transcription factor Cta4. I show that Cta4 binds to the *YHB1* promoter in vivo as a homodimer and is necessary, but not sufficient, for nitric oxide, nitrate and thiocyanate induced expression of *YHB1*. Based on these data I propose a model in which Cta4 transcriptional activation is inhibited under non-inducing conditions by a negative regulator. Understanding the mechanism by which *C. albicans* senses and responds to nitric oxide, nitrate and thiocyanate remains a question for future research.

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Abbreviations

<i>CTA4, YHB1</i>	gene
Cta4, Yhb1	protein
CaYhb1	<i>Candida albicans</i> flavohemoglobin
ScYhb1	<i>Saccharomyces cerevisiae</i> flavohemoglobin
P _{CaYHB1}	<i>Candida albicans</i> flavohemoglobin promoter
P _{ScYHB1}	<i>Saccharomyces cerevisiae</i> flavohemoglobin promoter
<i>hmpA</i>	bacterial flavohemoglobin gene
<i>norVW</i>	bacterial nitric oxide reductase genes encoding a flavorubredoxin and NADH:(flavo)rubredoxin reductase
<i>fhbA</i>	<i>Aspergillus nidulans</i> flavohemoglobin gene
DPTA NONOate	dipropylenetriamine NONOate (nitric oxide donor)
DETA NONOate	diethylenetriamine NONOate (nitric oxide donor)
LPO	lactoperoxidase
MPO	myeloperoxidase
NORE	nitric oxide-responsive element, 12 bp sequence (TATTACCGTCGG) located 226-238 bp upstream of the <i>YHB1</i> start codon and necessary for nitric oxide induced expression of <i>YHB1</i> , binding site for Cta4
NOx	nitric oxide, nitrite, nitrate
oxyanion	oxygen containing ionic compound with the formula AxOy where A is a compound or element and O is oxygen

pseudohalide	compound that has physical properties identical to true halides
RNS	reactive nitrogen species
ROS	reactive oxygen species
SCN^-	thiocyanate
spp.	species
TAP	tandem affinity purification

Chapter 1: Introduction

1.1 *Candida albicans* inhabits humans

1.1.1 *Candida albicans* is typically a benign commensal organism in healthy individuals

Candida albicans is an obligate commensal organism found in the mouth, gastrointestinal tract, skin and genitourinary tract of most people. Colonization of a host by *C. albicans* can occur within weeks of birth, and carriage of multiple strains on a single individual at different sites can occur, however, carriage of particular strains is not fixed and can change during the host's lifetime (Kumamoto and Vines, 2005). *Candida albicans* is unique among other commensal microbes in that it is able to colonize very different niches in and on the body with varying nutrient availability, pH, carbon sources, transition metal availability, and oxygen and carbon dioxide tensions. *Candida* species including *Candida albicans* do not have a specific nutritional requirement that would restrict its ability to grow outside of a human host, yet non-host reservoirs of *Candida albicans* are not known to occur (Cooney and Klein, 2008). This suggests that there are multiple mechanisms and strategies that have evolved in *C. albicans*, different from other fungi, which enable successful host colonization and survival without being particularly virulent.

For most individuals, a healthy immune system and normal microbial flora are usually sufficient to prevent *Candida* overgrowth. However, even in otherwise healthy individuals, *Candida albicans* overgrowth can occur and is most commonly manifested as an oropharyngeal infection or vaginitis (Calderone and Fonzi, 2001). These conditions

are not life threatening and usually clear up with the application of a topical antifungal treatment. One condition thought to contribute to the occurrence of *C. albicans* infections is a change in host microbial flora (Guarner and Malagelada, 2003). The disruption of microbial flora populations as a result of broad-spectrum antibiotic use leads to an increase in fungal burden that can result in superficial fungal infections and may even contribute to more chronic conditions such as asthma (Noverr et al., 2004; Noverr et al., 2005). Introducing microbial flora, e.g., probiotic lactic acid-producing bacteria, can serve as a therapy against fungal infection (Wagner et al., 1997; Meurman, 2005). The mechanism of this protection is unclear but is most likely a combination of introducing flora to compete for limited resources in the host and potentially an inhibition of growth due to antifungal compounds generated as secondary metabolites by competing microbes (Tampakakis et al., 2009).

1.1.2 *Candida albicans* is the most common human opportunistic fungal pathogen

The presence of *Candida albicans* on mucosal surfaces, in the mouth or intestines, does not indicate a disease state. However, in the case of immunologically compromised individuals this fungus can cause life-threatening invasive infections. *Candida* spp. have become problematic in hospitals where *Candida albicans* is the 4th most common cause of nosocomial bloodstream infections with a 40% mortality rate from invasive candidiasis (Wisplinghoff et al., 2004). Based on incident and mortality rates, it is estimated that in the US alone there are ~10,000 deaths a year from *Candida* infections (Sudbery et al., 2004). *Candida albicans* is the most common *Candida* spp. isolated in hospitals, accounting for more than half of all reported candidiasis cases. However,

emerging as agents of candidiasis are other *Candida* species including: *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, and *C. krusei* (Pfaller and Diekema, 2007). Factors that contribute to invasive candidiasis include the loss of competing microbial flora after antibiotic treatment, immunosuppressive therapy or chemotherapy, persistence of a prolonged inserted medical device such as catheters, autoimmune disorders and diseases. (Holley et al., 2009). *C. albicans* transitions from a commensal to an opportunistic pathogen as a result of a weakened immune system and loss of competing microbial flora; however, it is unclear how this transition occurs at the transcriptional level. Whether there is a distinct switch from commensal to pathogen, if it is a result of change in dynamic interplay between host and yeast, or whether the switch in *C. albicans* is always on and just needs the inhibiting action of the host immune system removed, is unclear (Hube, 2004).

One of the key factors in virulence is the ability to switch from yeast to hyphal form. A number of different environmental cues activate hyphal specific gene regulation including: growth at 37 degrees, neutral pH, presence of serum, 5% CO₂ concentration (partial pressure of CO₂ in blood) (Gow et al., 2002). The formation of hyphae allows *C. albicans* to penetrate tissue to escape immune cell capture. While the formation of hyphae is necessary for virulence (Carlisle et al., 2009), the yeast form is necessary for dissemination through the blood stream for complete systemic infections (Uppuluri et al., 2010).

Systemic *C. albicans* infections are treated using four main classes of antifungal drugs: polyenes, azoles, echinocandins and the fluorinated pyrimidine analogue 5-fluorocytosine (5-FC) (Cannon et al., 2007). Polyenes, such as amphotericin B, are large

amphipathic molecules that interact with membrane sterols resulting in disruption of membrane integrity (Ghannoum and Rice, 1999). Polyenes are very effective with few cases of clinical resistance. However, because these compound are cytotoxic against any organism with sterols in the outer membrane, polyenes can be toxic to mammalian cells and therefore have limited use in treating systemic infections (Odds et al., 2003).

5-FC is internalized by cells via a cytosine permease and subsequently converted to uracil and thymidine analogs that can disrupt protein synthesis and DNA replication. However, as a result of its narrow antifungal targets (*Candida* spp. and *Cryptococcus neoformans*) and high rates of resistance in clinical isolates (Stiller et al., 1982) 5-FC is typically only used in combination with other antifungals and rarely as the primary therapy.

Echinocandins, such as caspofungin, work by inhibiting the (1,3) D- β -glucan synthase, that synthesizes the main structural component of the fungal cell wall. Clinical resistance has remained relatively rare, but there have been clinical isolates identified from patients that stopped responding to caspofungin treatment (Odds et al., 2003). In these strains, mutations in *GSC1*, encoding beta-1,3-glucan synthase, are correlated with the reduced susceptibility to caspofungin (Park et al., 2005).

Azoles are the most widely used class of antifungals and work by inhibiting the synthesis of ergosterol, an important membrane component, connected to membrane fluidity and permeability. Azoles work by binding to the iron in the heme pocket of a cytochrome P450 mono-oxygenase, the lanosterol 14- α -demethylase, which functions as the rate limiting step in the biosynthetic pathway which converts Acetyl-CoA to ergosterol (Henry et al., 2000). Resistance to azoles can occur via a number of

different molecular mechanisms. These include mutations in the Erg11p binding pocket that prevent azole binding but not substrate binding (Orozco et al., 1998), overexpression of Erg11p (Morschhäuser, 2010), and the most common cause of azole resistance in clinical isolates is the increased expression of plasma membrane efflux pumps, major facilitator superfamily (MFS) transporters and ATP-binding cassette (ABC) pumps (Cannon et al., 2009).

1.2 Host immunity inhibits overgrowth of *C. albicans* by generating small antimicrobial compounds and peptides

1.2.1 Neutrophils and macrophages generate antimicrobial reactive nitrogen species (RNS) and reactive oxygen species (ROS)

The innate immune system is the first line of defense against invading pathogens. The professional phagocytic cells of the innate immune system, monocytes/macrophages and neutrophils, help protect the host by phagocytizing invading microorganisms and generating reactive oxygen (ROS) and reactive nitrogen species (RNS) to damage the captured organisms. Reactive oxygen species are generated by the NADPH phagocyte oxidase complex. NADPH oxidase complex is comprised of five subunits; two membrane proteins, and three cytosolic proteins. This system is assembled and activated by the low molecular weight GTP-binding protein Rac1 (in monocytes) or Rac2 (in neutrophils) (Zhao et al., 2003). When activated, this complex facilitates the transfer of electrons from NADPH to molecular oxygen to form superoxide (O_2^-) which then can diffuse to the phagosome or into the extracellular space (Fang, 2004). Superoxide can be readily converted to hydrogen peroxide in a pH and concentration dependent, non-

enzymatic process, or by superoxide dismutase (Fang, 2004). Activation of NADPH oxidase is also called respiratory burst because when activated the NADPH complex converts large amount of molecular oxygen into superoxide. The respiratory burst occurs rapidly when activated because all of the necessary protein components of the system are highly expressed in neutrophils and activation of the complex happens post-translationally (Nathan and Shiloh, 2000).

The targets of oxidative stress cover a broad spectrum of molecules and functions. Reactive oxygen species interact with numerous cellular targets and exert antimicrobial effects by inactivating metal centers, disrupting cellular respiration, inhibiting DNA replication by modifying bases or sugars, which results in DNA strand breaks, and by peroxidation of lipids (Slauch, 2011). Specific cellular targets and damage by reactive oxygen species is dependent on the concentration and redox state of the reactive oxygen species (Fang, 2004). DNA damage by ROS is dependent on the presence of iron which generates hydroxyl radicals ($\cdot\text{OH}$) from H_2O_2 via the Fenton reaction in which iron-containing proteins release free iron and this free ferrous iron is then oxidized by H_2O_2 to generate ferric iron and a hydroxyl radical (McCormick et al., 1998).

In addition to reactive oxygen species, reactive nitrogen species (RNS) are generated by phagocytic cells (MacMicking et al., 1997). Nitric oxide is generated in phagocytic cells by inducible nitric oxide synthase (iNOS), one of the three isoforms of nitric oxide synthase. All nitric oxide synthases generate nitric oxide by converting L-arginine into nitric oxide and L-citrulline in an oxygen- and NADPH-dependent manner (Fang, 2004). The other two forms of nitric oxide synthase, endothelial (eNOS) and neuronal (nNOS), generate very small amounts of nitric oxide (Stuehr, 1999). At low

concentrations nitric oxide serves as a signaling molecule regulating vascular tone and angiogenesis in epithelial tissue. One sensor of nitric oxide in mammalian cells is soluble guanylate cyclase (sGC) which responds to nitric oxide levels as low as 5 nM (Boon and Marletta, 2005). Binding of nitric oxide activates sGC which catalyzes the conversion of GTP to cGMP. cGMP acts as a second messenger regulating Ca^{2+} channels, protein kinases and phosphodiesterases. This second messenger activation allows transmission of the regulatory signal using nontoxic levels of nitric oxide (Denninger and Marletta, 1999).

In addition to its role as a signaling molecule, nitric oxide can serve as an effective antimicrobial compound. In phagocytic cells iNOS produces nitric oxide at a much higher concentration, up to 57 μM (Tillmann et al., 2011). However, unlike NADPH oxidase, iNOS is regulated at the transcriptional level via specific microbial recognition receptors, or by proinflammatory cytokines (IL-1 β , TNF- α and IFNs) (Fang, 2004). Nitric oxide readily diffuses through both water and the lipid bilayer of a cell. Cellular targets of nitric oxide are similar to those of ROS such as DNA, thiols, lipids and metal clusters of metalloproteins (Bowman et al., 2011). Nitric oxide also reacts with O_2^- to form peroxynitrite (ONOO^-), a potent oxidizing agent; this reaction occurs at near diffusion limited speed and does not require a catalyst (Missall et al., 2004). Peroxynitrite reacts readily with tyrosine residues and is highly microbicidal (Beckman and Koppenol, 1996). The physiological relevance of peroxynitrite formation is unclear because, in response to microbial invasion, NADPH oxidase is activated immediately. However, cellular iNOS activity is regulated at the transcriptional level and in macrophages there is an 8 hour lag from initial stimulation until nitric oxide generation

(Nathan and Shiloh, 2000). The temporal separation of superoxide and nitric oxide formation suggests peroxynitrite may not be as readily formed in vivo.

The effectiveness of ROS and RNS molecules generated by phagocytic cells is exemplified by results obtained from experiments on microbe-phagocyte co-cultures or direct treatment of microbes with ROS or RNS (Nathan and Shiloh, 2000). However, the most compelling evidence for the importance of these antimicrobial systems in host defense is seen in genetic disorders of innate immune cell function. The importance of NADPH oxidase can be seen in patients with chronic granulomatous disease (CGD) (Andrews and Sullivan, 2003). These patients have defects in genes that encode for one of the subunits of the NADPH oxidase. Patients with CGD have shorter life expectancies, and are more prone to chronic microbial infections (Andrews and Sullivan, 2003). No cases of iNOS defects have been identified in humans (Fang, 2004), but iNOS knockout mice are more susceptible to a number of pathogenic microbes including *Mycobacterium tuberculosis*, *Salmonella enterica* and *Staphylococcus aureus* (Nathan and Shiloh, 2000).

Even though ROS and RNS species both target similar cellular targets to exert their antimicrobial effect, evidence suggests that these mechanisms work synergistically and not redundantly. Mice that lack both iNOS and NADPH oxidase function develop spontaneous infections much more readily than mice lacking only one of these antimicrobial mechanisms (Shiloh et al., 1999). These mice also develop spontaneous infections from commensal bacteria unless treated continually with antibacterial and antifungal drugs (Shiloh et al., 1999). There is also evidence that suggests the ROS

system is important for defending against initial microbial invasion while the RNS system is necessary for clearing latent infections (Stenger, 1996).

1.2.2 Myeloperoxidase and lactoperoxidase catalyze the oxidation of halides by H_2O_2 to generate toxic hypohalous acids

Generation of antimicrobial compounds is not limited to ROS and RNS.

Peroxidases generate oxidants by transferring electrons from hydrogen peroxide to oxidize halides and the pseudohalide thiocyanate (SCN^-) to form hypohalous acids (Rada and Leto, 2008). Peroxidases come in two major classes: myeloperoxidase (MPO) and lactoperoxidase (LPO). Myeloperoxidases are found in neutrophils (up to 5% of dry weight is MPO) and monocytes but not differentiated macrophages (Rada and Leto, 2008). Chloride, bromide, iodide and the pseudohalide thiocyanate can all be oxidized by myeloperoxidase using hydrogen peroxide generated by dismutation of O_2^- by the NADPH oxidase system (Ihalin et al., 2006). This reaction generates strong oxidants, such as hypochlorite (OCl^-), that chlorinates phenols and amines, and disrupts glucose metabolism, respiration, and DNA replication (Aratani et al., 2002). Deficiency in myeloperoxidase activity is linked to an increase in susceptibility to *C. albicans* infections in both mice and humans (Cech et al., 1979; Parry et al., 1981). Mice deficient in both MPO and NADPH oxidase function show similar susceptibility to fungal invasion compared with the mice lacking only NADPH oxidase function (Missall et al., 2004). These results support a model in which the hydrogen peroxide substrate for chloride oxidation by MPO is derived from the superoxide generated by NADPH oxidase (Andrews and Sullivan, 2003).

The other major peroxidase is lactoperoxidase (LPO) which is generated by non-immune cells. Lactoperoxidase catalyzes the oxidation of SCN^- , Br^- , I^- , but not Cl^- to form hypohalic acids (Ihalin et al., 2006). LPO is found in saliva, milk and airway surface secretions (Xu et al., 2009). The hydrogen peroxide substrate for LPO is generated by two dual oxidase enzymes (Duox1, Duox2) generated by non-immune cells and expressed on epithelial surfaces along the digestive tract and in saliva and airway secretions (Donkó et al., 2005). In the mouth, lactoperoxidase is secreted in the saliva by submandibular glands and parotid glands and is referred to as salivary peroxidase (SPO) even though both LPS and SPO are coded by the same gene (Ueda et al., 1997). Myeloperoxidase is also released into the oral cavity as a result of neutrophil migration from the blood (Rada and Leto, 2008). Thiocyanate is the preferred substrate for MPO (van Dalen et al., 1997). Under conditions similar to that of saliva, even though concentrations of chloride are ten times the concentration of thiocyanate, the oxidant hypothiocyanite (OSCN^-) is the major product formed (Thomas and Fishman, 1986). Whereas in blood, because the concentration of chloride (100-140 mM) is far greater than thiocyanate (20-120 μM), hypochlorite is the major oxidant formed (Thomas and Fishman, 1986).

The importance of mucosal derived peroxidases and hydrogen peroxide appear to be important from an early age because 6-month-old infants generate the equivalent amount of lactoperoxidase as an adult in addition to receiving it through breast milk (Donkó et al., 2005). The combination of thiocyanate, hydrogen peroxide and lactoperoxidase under physiological conditions is sufficient for killing of *Candida albicans* and *Streptococcus* spp. in vitro. (Welk et al., 2009).

1.2.3 Antimicrobial peptides are short peptides that are important for host immune defense

Antimicrobial peptides are short (<60 amino acids) peptides that show highly specific antibacterial and antifungal activity with no toxicity to human epithelial cells (Zasloff, 2002). Antimicrobial peptides found in saliva are made by both professional immune cells (neutrophils) and non-immune epithelial cells. These peptides are essential for maintaining a healthy oral microbiota and can be considered part of the innate immune system along with salivary peroxidases, lysozyme, and mucins (Dale and Fredericks, 2005). The primary human oral antimicrobial peptides are: α - and β -defensins, LL-37 and histatins (Dale and Fredericks, 2005). These peptides show a broad range of antimicrobial activity with α - and β -defensins, and histatins all showing significant anti-fungal, specifically, candidacidal activity. The role of histatin 5 in *C. albicans* specific killing is the best studied of all the antimicrobial peptides (Kavanagh and Dowd, 2004).

Histatins are small, 3-4 kDa, peptides excreted by parotid and submandibular glands (Dale and Fredericks, 2005). Histatin 5 is 24 amino acids in length and the most effective of the 12 histatins at killing *Candida albicans* in vitro. After internalization, histatin 5 induces non-lytic release of ATP and causes the formation of ROS and cell cycle arrest at the G1 phase (Brogden, 2005). Membrane-specific binding of histatin 5 to Ssa1/2 (heat shock protein 70) in the cell wall results in the internalization of the toxic peptide by translocation and this internalization is essential for its candidacidal activity (Li et al., 2003).

1.2.4 Host microbial flora inhibits the overgrowth of *C. albicans*

The innate immune response defends against invading pathogens. In addition to a healthy immune system, control of infection by invading microbes and overgrowth of colonizing microbes (including *C. albicans*) requires a normal microbial flora (Hube, 2004). Perturbations of the microbial flora can arise as a result of broad-spectrum antibiotic use with yeast overgrowth on mucosal surfaces being one of the most common side effects of antibiotic treatment. Prevention of *C. albicans* overgrowth by commensal bacteria is mediated by different mechanisms. The first is simply competing for the same nutrients and habitable space in a given niche. Reducing the number of bacteria during antibiotic treatment means less competition for the same resources (Noverr and Huffnagle, 2004). Mice, which are not a natural reservoir for *Candida albicans*, can be experimentally colonized with this fungus only after successful antibiotic treatment (Clark, 1971; Kumamoto, 2011). The other is the direct interaction between bacteria and yeast. *P. aeruginosa* blocks *C. albicans* from forming hyphae by secreting a compound that mimics the *C. albicans* quorum sensing molecule farnesol that promotes the yeast state (Hogan et al., 2004). *Pseudomonas aeruginosa* binds to *C. albicans* and secretes virulence factors that kill *C. albicans*, but only when *C. albicans* is in the hyphal state (Hogan and Kolter, 2002). *Lactobacillus* spp. have been shown to prevent the overgrowth of *C. albicans* in vivo and prolong the survival and reduce the severity of mucosal infections (Wagner et al., 1997). *Lactobacillus* spp. and *Bifidobacterium* spp. have begun to be used in probiotic therapy (Meurman, 2005). The exact mechanism by which this occurs is not clear, but it is most likely a combination of competition for

resources and lactic acid production which lowers the pH of the surrounding microenvironment (Köhler et al., 2012).

1.3 Microbiota generated acidified nitrite is an important innate defense mechanism against *Candida albicans* and other pathogens

1.3.1 Commensal bacteria reduce nitrate to nitrite in the oral cavity

In the body there are two primary sources of nitrite: diet and nitrate reduction (Gladwin, 2004). Vegetables and cured meats are the primary dietary sources of nitrite (Hord et al., 2009). A more abundant source of nitrite is via the reduction of nitrate to nitrite by oral anaerobic bacteria (Lundberg et al., 2008). For metabolic purposes, oral bacteria utilize nitrate as an electron acceptor and reduce it to nitrite. The primary site of nitrate reduction is at the middle and rear of the tongue with some nitrate reduction at the front of the tongue (Doel et al., 2005). Nitrate reduction on the tongue is carried out by both strict anaerobes, *Veillonella* spp. and facultative anaerobes *Actinomyces* and *Rothia* spp. (Doel et al., 2005). In that sense, the level of nitrite is directly correlated to salivary nitrate concentrations and active oral microbial flora.

The primary source of nitrate is through diet, mainly the consumption of uncooked vegetables (Hord et al., 2009). Nitrate is also generated endogenously: nitric oxide synthases generate nitric oxide from arginine; this nitric oxide is eventually converted to nitrate by hemoglobin. Nitrate ingested through diet is absorbed into the bloodstream from the gut and circulates in plasma (Sobko et al., 2005). Plasma nitrate is excreted in the urine and sweat, however, up to 25% of nitrate is reabsorbed by salivary glands to generate concentrations in saliva 10-20 fold higher than in plasma (Weitzberg

and Lundberg, 1998). The daily flow of nitrate through the oral cavity is significant as the average adult swallows 1-1.5 liters of saliva daily (Gladwin et al., 2005). It is unclear why nitrate is specifically concentrated in the saliva. Along with nitrate, thiocyanate and iodide are actively concentrated in saliva and evidence suggests that all these compounds share the same transport mechanism in the salivary gland (Tenovuo, 1989).

1.3.2 Acidified nitrite is an effective antimicrobial agent

Nitrite is generated from salivary nitrate by nitrate-reducing oral bacteria. In the neutral pH of the oral cavity, nitrite is not toxic even at high concentrations. As that nitrite is swallowed and enters the acidic gastric juices, it is rapidly broken down to toxic nitrogen oxide compounds including nitric oxide (Lundberg et al., 2004). The high acidity of the gut alone has been shown to be effective in killing some microbes (Rao et al., 2006). The pH of gastric juice of the stomach can be as low as 1.5-2.5 in healthy individuals (Smith, 2003). At acidic pH, nitrite is protonated to form nitrous acid ($pK_a = 3.2$). Nitrous acid spontaneously breaks down to dinitrogen trioxide and then to nitric oxide and nitrogen dioxide (Figure 1-1). All of the products and intermediates in the conversion of acidified nitrite all have potential to disrupt biological processes (Bowman et al., 2011).

The most recognized product of nitrite acidification is nitric oxide. Nitric oxide is a free radical with one unpaired electron that reacts readily with iron-sulfur groups, unsaturated lipids, amines, and reacts readily with O_2 to form N_2O_3 , a strong electrophile that reacts with primary amines leading to deamination of DNA bases (Caulfield et al., 1998). Nitric oxide can also disrupt protein function by disrupting iron/zinc-sulfur clusters and causing nitration of tyrosine residues (Beckman and Koppenol, 1996).

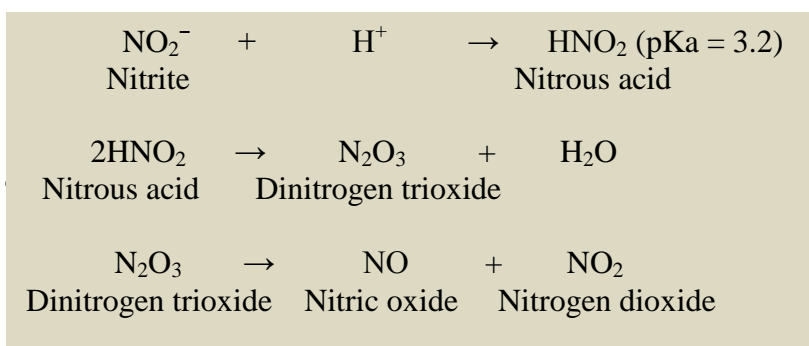


Figure 1-1. Intermediates and products of nitrite acidification

Nitrite is non-enzymatically converted to nitric oxide and nitrogen dioxide at acidic pH (pKa = 3.2). Nitrite acidification generates dinitrogen trioxide, a powerful nitrosating agent that is highly electrophilic. Dinitrogen oxide disassociates into nitric oxide and nitrogen dioxide. Figure adapted from Lundberg et al., 2004.

Nitrogen dioxide (NO₂) is formed in equal stoichiometric quantities from the acidification of nitrite. NO₂ is a free radical oxidant that reacts with various functional groups of biological significance including thiols, fatty acids, and tyrosine (Bowman et al., 2011). The conversion of nitrite to other nitrogen oxide species is pH dependent. In addition to pH, reducing agents such as ascorbic acid and glutathione drive the reaction toward nitric oxide formation (Fite et al., 2004).

1.3.3 *C. albicans* is highly resistant to acidified nitrite

At neutral pH nitrite has little effect on microbial growth and can even help growth of microbes that use nitrite as an electron acceptor in oxygen free media or as a nitrogen source (Cove, 1979; Lundberg et al., 2004). However, with increasing acidity the antimicrobial effects of nitrite are effective against a broad array of bacteria and fungi. Acidified sodium nitrite is antimicrobial against a broad range of tested organisms; however some species are more resistant than others. *Candida albicans* is more resistant to the effects of acidified nitrite compared to other tested *Candida* species as well as other opportunistic pathogenic fungi including *Cryptococcus neoformans* and *Aspergillus fumigatus* (Anyim et al., 2005). *C. albicans* is more resistant to the effects of acidified nitrite than bacterial oral pathogens such as *Streptococcus mutans*, *Porphyromonas gingivalis*, and *Capnocytophaga gingivalis* (Xia et al., 2006) as well as other common pathogens such as *Trichophyton rubrum*, *Staphylococcus aureus* (Weller et al., 2001), *Salmonella typhimurium*, *Shigella sonnei*, and *Escherichia coli* O157 (Dykhuizen et al., 1996). Pre-exposure of *E. coli* to acidified sodium nitrite confers greater resistance to the effects of acidified sodium nitrite compared to untreated controls,

indicating an inducible response mechanism to counteract the toxic effects of acidified nitrite (Bower et al., 2009).

1.4 Defense mechanisms against RNS, including nitric oxide and acidified nitrite

1.4.1 Bacteria respond to nitrosative stress by upregulation of nitric oxide detoxifying enzymes

Nitric oxide is formed as a by-product during the reduction of nitrate to N₂ or ammonia in microbes (Ye et al., 1994). High concentrations of nitric oxide, generated by immune cells in response to microbes and cytokine activation, is a potent inhibitor of essential cellular processes and is cytotoxic (Stuehr and Nathan, 1989). In response to nitric oxide and other RNS production, commensal and pathogenic microbes have evolved strategies to counteract nitrosative stress. In three different microarray analyses of *E. coli* gene expression in response to different nitrogen oxide sources (GSNO, NO gas, acidified nitrite) in complex or defined media, under aerobic or anaerobic conditions, only three genes were upregulated under all three experimental conditions: *hmpA*, *norV* and *norW* (Spiro, 2006). Both *hmpA* and the *norVW* complex are able to enzymatically convert nitric oxide into either nontoxic nitrate or nitrous oxide.

1.4.1a Hmp is a nitric oxide dioxygenase (NOD)

The most extensively studied and fully understood mechanism of nitric oxide detoxification is the dioxygenase activity of flavohemoglobin proteins that convert nitric oxide to nitrate in an oxygen dependent manner ($2\cdot\text{NO} + 2\text{O}_2 + \text{NAD(P)H} \rightarrow \text{NAD(P)}^+ + \text{H}^+ + 2\text{NO}_3^-$) (Gardner, 2005). Flavohemoglobins consist of three highly conserved domains, a globin domain, an FAD binding domain followed by a C-terminal NAD(P)H

binding domain. Flavohemoglobins are nearly ubiquitous in microorganisms and have been shown to be important in nitrosative stress tolerance and important for full virulence of some pathogenic microbes (Forrester and Foster, 2012). In *E. coli*, the flavohemoglobin gene *hmpA* is strongly induced by nitric oxide (Poole et al., 1996) and cells lacking *hmpA* have increased sensitivity to nitric oxide (Gardner et al., 1998).

hmpA expression is controlled by two negative regulators of gene expression, NsrR and Fnr, and one positive regulator, MetR. Fnr is a global regulator that regulates fumarate and nitrate reduction. Active Fnr contains a $[4\text{Fe-4S}]^{2+}$ cluster and regulates over 100 genes, by repression or activation, in response to O_2 availability (Crack et al., 2008). Fnr represses *hmpA* expression in the absence of oxygen or nitric oxide by actively binding to the promoter (Cruz-Ramos et al., 2002). Under anaerobic conditions nitric oxide reacts with the Fnr $[4\text{Fe-4S}]$ cluster to form an inactive dinitrosyl-iron-cysteine complex $[\text{Fe}(\text{NO})_2(\text{Cys})_2]$, which causes loss of DNA binding and thus derepression of *hmpA* expression. However, in strains lacking *fnr*, nitrite is still able to induce *hmpA*, indicating the presence of additional components of *hmpA* regulation (Poole et al., 1996).

A second negative regulator called NsrR binds to the *hmpA* promoter to regulate transcription (Tucker et al., 2010). Like Fnr, NsrR contains an iron-sulfur cluster and senses nitric oxide directly (Tucker et al., 2008). NsrR regulates genes involved in nitric oxide resistance, general stress response and iron-sulfur cluster assembly. It is unclear exactly how NsrR senses nitric oxide – whether it is through a $[4\text{Fe-4S}]$ or $[2\text{Fe-2S}]$ cluster (Fleischhacker and Kiley, 2011). While the exact state of the iron-sulfur complex in vivo is uncertain, the general mechanism is proposed to be the destabilization of the

Fe-S complex, formation of a dinitrosyl iron cysteine with decreased affinity for target DNA. Release of NsrR from DNA binding sites results in derepression of target genes (Tucker et al., 2010).

MetR, a regulator of methionine biosynthesis, is the third transcription factor that regulates expression of *hmpA*. MetR binds to the intragenic region between *glyA-hmpA*, which are divergently transcribed (Membrillo-Hernández et al., 1998). *glyA* encodes a serine hydroxyl-methyltransferase. Homocysteine is a cofactor of MetR and when bound to MetR, inhibits the binding of MetR to the *glyA-hmpA* intragenic region (Spiro, 2007). Treatment of cells with the nitric oxide donor GSNO (S-Nitrosoglutathione) results in nitrosylation of homocysteine which leads to inactivation of homocysteine and loss of binding to MetR. This leads to binding of apo-MetR to the *hmpA* promoter, resulting in the activation of *hmpA* transcription (Membrillo-Hernández et al., 1998). This activation of *hmpA* transcription in response to GSNO is lost by adding homocysteine to the media or in MetR mutant strains. However, MetR-dependent activation of *hmpA* expression has not been shown to occur using gaseous nitric oxide, which nitrosates homocysteine less efficiently than GSNO (Spiro, 2007).

1.4.1b *norVW* genes encode a nitric oxide reductase (NOR)

Under anaerobic conditions, *hmpA* shows negligible nitric oxide dioxygenase activity (Gardner and Gardner, 2002). Nitric oxide detoxification under anaerobic conditions requires the NorR regulated genes *norV* and *norW*. NorV is a flavorubredoxin that has a di-iron center and interacts with NorW, an NADH:flavorubredoxin oxidoreductase (Gardner et al., 2003). Together these gene products effectively convert nitric oxide to nontoxic nitrous oxide in an oxygen independent manner. The nitric oxide

reductase activity of the NorVW system is restricted to strictly anaerobic or microaerobic conditions and this activity is reduced in the presence of oxygen (Poole, 2005).

norVW is regulated by the NO-responsive, DNA-binding transcriptional activator NorR. NorR is comprised of three distinct functional domains: an N-terminal GAF domain that binds a monomeric non-heme iron, a AAA⁺ ATPase domain, and a C-terminal, helix turn helix, DNA-binding domain (Tucker et al., 2006). Induction of *norVW* transcription in response to nitric oxide is NorR dependent. In the absence of nitric oxide, NorR-Fe(II)-DNA complexes bind to the *norVW* promoter at three distinct locations (Justino et al., 2005). In the absence of nitric oxide, the GAF domain represses ATPase activity of the AAA⁺ domain by intramolecular repression. Upon exposure to nitric oxide Nor-Fe(II) is converted to a NorR-{Fe(NO)}⁷ complex. This results in the derepression of the AAA⁺ ATPase domain and enables transcriptional activation of the *norVW* operon (D'Autr aux et al., 2005). The role of the GAF domain as a transcriptional repressor is supported by the observation that a GAF domain deletion NorRΔGAF results in constitutive activation of a *norV-lacZ* reporter gene in *E. coli* (Tucker et al., 2008). Further characterization of the GAF domain has identified point mutants that either fail to bind iron in vivo (D99A) resulting in loss of induction by nitric oxide, and point mutants that constitutively express *norV* (Tucker et al., 2008).

1.4.2 Fungi detoxify nitric oxide primarily by upregulating flavohemoglobin genes

The fungal response to nitrosative stress is less well characterized compared to the bacterial response, but just as in bacteria, the highly conserved flavohemoglobins provide a critical defense against nitrosative stress by converting nitric oxide to nitrate.

Flavohemoglobins are found in >90% of the sequenced, or partially sequenced genomes of fungi in the phylum Ascomycota (Hoogewijs et al., 2012). This includes species in the genus *Aspergillus*, *Candida*, and *Saccharomyces*. Globin genes are less well represented in the phylum Basidiomycota (60%) which includes the human opportunistic pathogen *Cryptococcus neoformans* (Hoogewijs et al., 2012). Flavohemoglobins are highly conserved throughout the microbial community. The *E. coli* flavohemoglobin Hmp shares a 38% sequence identity with the *S. cerevisiae* flavohemoglobin Yhb1 including highly conserved residues in the heme binding pocket, FAD binding domain and NADPH binding domain (Tillmann et al., 2011).

The first fungal flavohemoglobin identified was the *S. cerevisiae* Yhb1 flavohemoglobin in 1953, but its function remained unknown for nearly fifty years (Keilin, 1953). Shortly after the identification of Hmp as a nitric oxide detoxifying enzyme in *E. coli*, Liu et al. showed deletion of the *S. cerevisiae* *YHB1* gene results in hypersensitivity to nitric oxide and a *yhb1Δ* deletion mutant had no nitric oxide-consuming activity (Liu et al., 2000).

Flavohemoglobins in fungi have been shown to be induced by nitric oxide and to protect against nitrosative stress (Forrester and Foster, 2012). In many fungi able to cause infection in humans, the flavohemoglobin gene has been identified as a significant virulence factor in mouse models of infection (de Jesús-Berríos et al., 2003). In some fungal species, including *Aspergillus oryzae* (Zhou et al., 2009) and *C. albicans* (Ullmann et al., 2004), there is more than one gene encoding a flavohemoglobin protein. In these two species only one of the flavohemoglobin gene products is necessary for full protection against nitric oxide stress.

Along with flavohemoglobins, denitrifying fungi have reductases that convert nitric oxide to nitrous oxide. Denitrification involves the reduction of nitrate to nitrous oxide when oxygen, the favored electron acceptor, is unavailable. Cytochrome P450-nor is a soluble monoheme protein that reduces nitric oxide to nitrous oxide by transferring a pair of electrons from NAD(P)H, without a flavin coenzyme intermediate, directly to the P450-nor heme and onto nitric oxide ($2\text{NO} + 2\text{e}^- \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O}$) (Nakahara et al., 1993). Cytochrome P450-nor (“nor” is used to distinguish it from other P-450 proteins that catalyze mono-oxygenase reactions of organic substrates) activity is greatest under denitrification conditions: limited oxygen in the presence of nitrate (Zumft, 1997). Nitric oxide reductases have been identified in a number of denitrifying fungi including the opportunistic human pathogen *Histoplasma capsulatum*. *H. capsulatum* is inhaled and spreads infection within a host by multiplying within naïve macrophages (Nittler et al., 2005). Activated macrophages generate nitric oxide which inhibits *H. capsulatum* replication. *H. capsulatum* is able to withstand the toxic effects of nitric oxide and remain dormant in an infected host over many years. The *Histoplasma* genome does not code for a flavohemoglobin homolog (Nittler et al., 2005). Instead *Histoplasma* detoxifies nitric oxide by a P450-nor that is induced by nitric oxide, experimentally induced by the addition of the nitric oxide releasing compound DPTA NONOate (Nittler et al., 2005).

1.4.3 *C. albicans* genome codes for three flavohemoglobin orthologs

The *Candida albicans* genome codes for 3 genes, *YHB1*, *YHB4* and *YHB5* whose predicted amino acid sequences all have between a 25-31% sequence similarity to the *S. cerevisiae* Yhb1 protein (Ullmann et al., 2004). Of these three *C. albicans* genes, only

deletion of the *YHB1* gene results in both a loss of nitric oxide consuming activity and an increased sensitivity to the nitric oxide generating compounds NOC-18 and sodium nitrite (Ullmann et al., 2004). The *YHB4* and *YHB5* gene products have no known function and it is unknown if either of these two genes codes for a functional flavohemoglobin with dioxygenase activity able to convert nitric oxide to nitrate. *YHB1* is transcriptionally activated in response to sodium nitrite, nitric oxide gas and NOC-18 (Ullmann et al., 2004), but is not induced by oxidative, osmotic or thermal stress (Enjalbert et al., 2003). In two independent studies, deletion of the *C. albicans* *YHB1* gene resulted increased sensitivity to nitric oxide releasing compounds and attenuated virulence in a mouse model of disseminated candidiasis (Ullmann et al., 2004; Hromatka et al., 2005).

Genome-wide microarray analysis was used to identify gene expression in response to cells treated with the nitric oxide donor DPTA NONOate (Hromatka et al., 2005) and gene expression from cells cultured with activated mammalian macrophages (Lorenz et al., 2004). Under both conditions only *YHB1* transcript levels, not *YHB4* or *YHB5* transcripts, were significantly induced. Global microarray analysis of *C. albicans* gene expression identified 65 genes that were upregulated (2-25 fold) after 10 minute exposure to DPTA NONOate (Hromatka et al., 2005). After a 40 minute exposure, most of these genes returned to uninduced levels of expression with only 9 genes, including *YHB1*, still highly expressed after a 2 hour exposure to DPTA NONOate (Hromatka et al., 2005). The reason for this sustained induction is not known.

In fungi no known nitric oxide receptor has been identified. However, in *C. albicans*, transcriptional activation of *YHB1* by nitric oxide has been shown to be

mediated by the fungal-specific, zinc cluster transcription factor Cta4 (Chiranand et al., 2008). Mutational analysis of the *YHB1* upstream regulatory region identified a 12-base pair region as a nitric oxide-responsive element (NORE) necessary for nitric oxide induction of *YHB1*. Disruption of the NORE results in loss of *YHB1* induction by nitrite (Chiranand et al., 2008). Affinity capture of proteins from whole cell extracts with an immobilized NORE-containing oligonucleotide identified five transcription factors that bind to the NORE in vitro. Of those five proteins, only the deletion of *CTA4* resulted in loss of *YHB1* induction by nitrite and increased sensitivity of cells to DPTA NONOate. Deletion of *CTA4* also mildly attenuated virulence in a mouse model of disseminated candidiasis (Chiranand et al., 2008).

The nitric oxide stress response has not been extensively studied in non-*albicans* species of *Candida*. Of the clinically relevant *Candida* spp, *C. albicans* accounts for greater than 50% of all reported blood stream infections (BSI). The non-*albicans* spp. *C. glabrata*, *C. parapsilosis*, *C. dubliniensis*, *C. krusei* and *C. tropicalis* make up the remainder of reported species that cause BSI (Pfaller and Diekema, 2007). All of these *Candida* spp. are susceptible to nitric oxide stress with *C. parapsilosis* and *C. krusei* being the most susceptible and *C. albicans* the most resistant of the common *Candida* spp (McElhaney-Feser et al., 1998).

1.5 Non-nitric oxide induced flavohemoglobin gene expression in microbes

Microorganisms up-regulate flavohemoglobin in response to nitric oxide. Flavohemoglobins effectively convert nitric oxide to nitrate using molecular oxygen as a

substrate. Microbial flavohemoglobin expression, however, is not solely regulated by nitrosative stress.

Bacterial flavohemoglobin and single domain globin expression is induced under both microaerobic and anaerobic growth conditions. The *E. coli* (Constantinidou et al., 2006), *B. subtilis* (LaCelle et al., 1996), and *Alcaligenes entrophus* (Cramm et al., 1994) flavohemoglobins are highly expressed when cultured in low oxygen. *hmpA* in *E. coli* is only slightly induced (~2 fold) by a shift from aerobic to anaerobic conditions. However, when grown anaerobically in the presence of nitrate, *hmpA* expression increases 20 fold (Poole et al., 1996). *B. subtilis* and *A. entrophus* are only able to grow anaerobically when nitrate is added to the medium (LaCelle et al., 1996; Cramm et al., 1994). Under aerobic conditions, however, nitrate does not significantly induce *hmpA* expression in *E. coli* (Poole et al., 1996).

In fungi the regulation of flavohemoglobin is somewhat different. At low oxygen tension, anaerobic or hypoxic growth, expression of both the *S. cerevisiae* *YHB1* (Zhao et al., 1996) and *C. albicans* *YHB1* (Setiadi et al., 2006) flavohemoglobin genes drops significantly. *S. cerevisiae* flavohemoglobin expression increases with increased oxygen concentration and is maximal at 100% oxygen tension (Crawford et al., 1995).

The filamentous fungi *Aspergillus nidulans* is an obligate aerobe with limited survival under anaerobic conditions (Willger et al., 2009). *A. nidulans*, unlike *C. albicans* or *S. cerevisiae*, is able to take up nitrate and reduce it to ammonium and incorporate this ammonium into biomass in a process called nitrate assimilation (Siverio, 2006). When *A. nidulans* is grown under conditions in which nitrate is the sole nitrogen source at normal oxygen tension, the flavohemoglobin gene *fhbA* is induced along with

nitrate assimilation genes (Schinko et al., 2010). Both *fhbA* and nitrate assimilation genes are regulated by the transcription factor NirA (Berger et al., 2006). It is unclear why the flavohemoglobin gene is induced during nitrate assimilation. *fhbA* expression may be a consequence of nitric oxide formation during nitrate reduction to protect against endogenously generated nitric oxide during nitrate reduction, however strains lacking a functional flavohemoglobin gene grow equally as well as wild-type strains under nitrate assimilating conditions (Schinko et al., 2010).

Flavohemoglobin expression is primarily induced by nitric oxide.

Flavohemoglobin expression can also be modulated by signals other than nitric oxide including nitrate and oxygen tension. In both bacteria and fungi it is not known whether nitrate directly induces flavohemoglobin expression or if nitric oxide generated as a byproduct during nitrate reduction is the signal that induces expression of flavohemoglobin genes.

Chapter 2: Materials and methods

2.1 Strains and culture growth conditions

The strains used in this study are listed in Appendix A. All *C. albicans* strains were cultured in rich liquid medium at 30°C, YEPD (2% peptone, 1% yeast extract, 2% dextrose), pH ~6.0. All *C. albicans* strains were stored in YEPD + 15% glycerol at -80° and subcultured on solid media (YEPD with 2% agar). For experiments done at specific pH, HCl or NaOH was added directly to YEPD the day of the experiment.

Transformants were plated on either YEPD + 200 µg/ml nourseothricin, or on synthetic media drop-out plates (6.7 g yeast nitrogen base, 20 g dextrose, 1.5 g drop-out mix).

S. cerevisiae wild-type laboratory strains were cultured in YEPD. Transformants were continually subcultured on synthetic media drop-out plates and grown in drop-out liquid media, supplemented with required nutrients. All growth of overnight cultures, for both *C. albicans* and *S. cerevisiae*, was done at 30° in glass culture tubes in a tissue culture rotator.

2.2 Genome-wide gene expression analysis

Growth conditions: Strain SN152 was grown overnight in YEPD at 30°. Next day cultures were diluted to a starting concentration of OD₆₀₀ = 0.2 in 1 liter of YEPD (pH ~6.0). Cells were grown for 4 hours, shaking 200 rpm at 30° C, to an OD₆₀₀ ~1.0, divided into 4 equal 250 ml cultures and treated with 5 mM of NaCl (control), 5 mM NaNO₂, 5 mM NaNO₃, 5 mM NaSCN or 0.02 mM DPTA NONOate (freshly dissolved in PBS buffered to pH 7.2). Cultures were incubated for an additional 10 minutes then

harvested by vacuum filtration onto Whatman® cellulose nitrate 0.45 µm membrane filters. The filters were immediately placed in individual 50-ml centrifuge tube, flash-frozen in liquid nitrogen and stored at -80°C.

RNA extraction: Total RNA was extracted using hot acidic phenol. Briefly, cells were resuspended in 12 ml AE buffer (50 mM NaOAc, pH 5.2, 10 mM EDTA), 12 ml acid phenol pH 4.3 (Sigma P4682) and 800 µl 25% SDS. Cells were incubated in a 65°C water bath for 20 minutes, vortexing every 5 minutes. Tubes were placed on ice for 5 minutes and then centrifuged 5 minutes in a tabletop centrifuge at 3500 RPM at 4°C. The aqueous phase was removed and placed in a new 50 ml tube and re-extracted with 12 ml acid phenol and then centrifuged in a tabletop centrifuge. The aqueous phase was placed in a 50 ml phase-lock tube with 12 ml chloroform, mixed by inversion and spun 5 minutes. Supernatant was transferred to a new tube with 1/10 volume 3M NaOAc, pH 5.2 and 1 volume isopropanol. Tubes were centrifuged for 10 minutes, supernatant decanted and the pellet was washed with 70% ethanol. The sample was centrifuged 5 minutes, ethanol decanted and the pellet dried by placing aluminum foil over open tubes and leaving tubes in 37°C incubator for 10 minutes. RNA was suspended in 2 ml DEPC-treated water at 4°C overnight.

Microarray: RNA was reverse transcribed to cDNA using oligo (dT) as a primer and each sample was labeled with Cy3 or Cy5 by aminoallyl labeling. *C. albicans* whole genome microarrays were created by printing 70-mer oligonucleotides, representing all verified and putative open reading frames, onto glass slides. Labeled cDNA from a treated sample were mixed with labeled reference sample (untreated) cDNA and hybridized to a single microarray slide. For each condition, a biological replicate was

generated and technical replicates for each biological replicate were performed by swapping labeling dyes for the reference and treated samples, so that for each condition four arrays were performed. Following hybridization each array was scanned using an Axon Instruments 4000B scanner. Data images were extracted using Axon Instruments GenePix program. For each array, the median Cy3 and Cy5 spot intensities were normalized so that total fluorescence in each channel was equal. For each array, the ratio of fluorescence intensity for the experimental condition (treated) and control condition (untreated) was calculated. Ratio values from each array for each condition (biological and technical replicates) were calculated and ratio values with a P values >0.05 were discarded. Ratio values were converted to log base 2 and visualized using TreeView (Eisen et al., 1998).

2.3 Analysis of *lacZ* gene expression in yeast using β -galactosidase assays

2.3.1 Yeast β -galactosidase assays

β -galactosidase assay were done as described by Chirinand et al. (Chiranand et al., 2008) with some modifications. Overnight cultures were used to inoculate 3 ml YEPD to a starting $OD_{600} = 0.08$. Cultures were grown in a tissue culture rotator for 4 hours at 30°C. After 4 hours cultures were treated with indicated compounds from stock solutions. Cultures were incubated for an additional hour and the OD_{600} of each culture was determined using a Spec20. 1 ml culture was pelleted in a microcentrifuge and resuspended in 0.8 ml Z-buffer with 2.7 μ l β -mercaptoethanol per 1 ml, 120 μ l chloroform and 80 μ l 0.1% SDS and vortexed. Samples were preincubated in a 37°C water bath for 5 minutes and then 56 μ l of a 13.55 mg/ml stock solution of chlorophenol

red- β -D-galactopyranoside (CPRG) diluted 1/10 in Z-buffer was added to each tube. Tubes were then incubated in 37°C water bath for 10 minutes. After 10 minutes the reaction was stopped by adding 400 μ l of 1 M Na₂CO₃, and cell debris was pelleted by centrifugation, top speed, for 5 minutes. The absorbance was read at 578 nm using 1 ml of supernatant from each sample to determine the amount of CPRG converted to chlorophenol red as a measure of β -galactosidase activity. Specific β -galactosidase activity for each culture was determined using the formula: $OD_{578}/[OD_{600} \text{ of culture} \times \text{reaction time in minutes}]$. For each experiment, activity was represented as fold change in expression relative to uninduced control. For each condition tested, three independent cultures were assayed on three different days (total of 9 data points) and data from each day was normalized to uninduced wild-type control.

In *S. cerevisiae* β -galactosidase assays were done as described above with the following modifications. Strains were grown overnight in selective drop out media to maintain plasmid selection. To set the expression of Cta4 through the TetR/tetO system, cultures were grown with indicated concentrations of tetracycline added directly from a 10 mg/ml stock solution of tetracycline dissolved in 100% ethanol. Overnight cultures were diluted to a starting $OD_{600} = 0.25$, grown for 4 hours at 30°C and treated with indicated compounds. Cultures were incubated for an additional hour and the OD_{600} of each culture was measured. 1 ml culture was pelleted and resuspended in 0.8 ml Z-buffer with 2.7 μ l β -mercaptoethanol per 1 ml buffer, 120 μ l chloroform, 0.1% SDS and CPRG were incubated in a 28°C water bath and monitored for color change (yellow to orange). After color change was detected, the reactions were stopped by adding 400 μ l of Na₂CO₃,

the time recorded and amount of CPRG converted to chlorophenol red was determined by measuring absorbance at 578 nm.

2.3.2 Construction of the 16xNORE-*YHB1-lacZ* reporter gene

β -galactosidase assays in *C. albicans* were done using a strain containing the pAU95-P_{*YHB1-lacZ*}(*Nou*^R) or pAU95-16xNORE-*YHB1-lacZ*(*Nou*^R) plasmid integrated at the *HWP1* locus. The former plasmid lacks the *YHB1* open reading frame (*YHB1* promoter driving *lacZ*) and the latter plasmid contains sixteen copies of the NORE (16xNORE) inserted into the 5' upstream regulatory region of the *YHB1* promoter at the location of the native NORE sequence, and shows ~4 fold higher maximum induction ratios than the native *YHB1-lacZ*. The pAU95-16xNORE-*YHB1-lacZ* plasmid was constructed by a former undergraduate in the lab Dorothy Koveal using the pAU95 plasmid (Uhl and Johnson, 2001). To give greater flexibility in introducing the plasmid into different *C. albicans* strains, I replaced the *URA3* selectable marker gene in pAU95 with the *C. albicans* optimized *caSAT1* (streptothricin acetyltransferase-*Nou*^R) gene from the plasmid pSFS1 (Reuss et al., 2004). Oligonucleotide primer sequences used for this construction are listed in Appendix B. Plasmids were digested with Kpn1 and either MscI (pAU95) or EcoRV (pSFS2) then ligated to generate the plasmid pAU96-16xNORE-*YHB1-lacZ*(*Nou*^R). The plasmid was linearized by cutting with PshAI and transformed by electroporation into the indicated *C. albicans* strains.

2.3.3 *S. cerevisiae YHB1* reporter gene construct

The *S. cerevisiae* 5' upstream regulatory region, ~900 bp upstream of the *YHB1* ORF, was amplified and ligated into plasmid pSEYC102 (do Valle Matta et al., 2001) which contains a truncated *lacZ* reporter gene. The final plasmid was a *URA3*

centromeric autonomous plasmid with the *S. cerevisiae* *YHB1* promoter driving *lacZ* expression ($P_{ScYHB1-lacZ}$).

2.4 qRT-PCR analysis of flavohemoglobin gene expression

C. albicans growth conditions: Strains grown overnight in YEPD were diluted into fresh YEPD media with a starting concentration of $OD_{600} = 0.2$. Cells were grown for 4 hours in an orbital shaker, and then the culture was divided into 20 ml aliquots, treated with indicated compounds and grown for 10 additional minutes. 10 ml of each culture was then poured into a 15 ml conical tube and centrifuged for 3 min at 2000 g and the cell pellet was flash frozen on dry ice and stored at -80°C . Total RNA was recovered using hot acid phenol extraction (Collart and Oliviero, 2001).

Aspergillus nidulans growth conditions: All *A. nidulans* growth and RNA extraction was done at the MD Anderson Cancer Center in the lab of Dr. Gregory S. May. Growth and treatment conditions to measure flavohemoglobin expression were done as described by Schinko et al. (Schinko et al., 2010). Briefly, strain R21 (yA2 paba A1) was grown overnight in glucose minimal media (GMM) with ammonium tartrate with essential vitamins at 37°C . Next day biomass was filtered with miracloth, washed with deionized water and incubated in GMM without a nitrogen source for 30 minutes. After 30 minutes the culture was divided into 5 equal cultures and treated with either 10 mM NaCl, 10 mM NaNO_2 , 10 mM NaNO_3 , 10 mM NaSCN or 1.5 mM DPTA NONOate for 30 min in 50 ml media. Mycelia were washed and frozen in liquid N_2 . Mycelia were broken apart by grinding and RNA was extracted by addition of Trizol.

cDNA synthesis: Total RNA was treated with recombinant DNase I, RNase free (Roche), for 15 minutes at 33°C for 15 minutes and heated at 75°C for 10 minutes to inactivate DNase I. cDNA was synthesized in a 20 µl reaction using 2 µg of total DNase I treated RNA as a template. RNA was reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions and oligo (dT)₁₅ as a primer. The RT-PCR product was diluted 1:100 in Milli-Q water. 5 µl cDNA was used for a 20 µl qPCR reaction using SYBR Green FastMix. cDNA, 2x SYBR master mix, ddH₂O and primer (400 nm final concentration) was mixed and threshold cycle value (C_T) values determined using an Applied Biosystem 7000 Sequence Detection System (ABI7000). For all reactions the actin gene (*C. albicans ACT1* or *A. nidulans actA*) was used as the housekeeping gene to normalize variation in expression values between experiments. The fold change in expression was determined using the delta-delta C_T method.

2.5 Growth inhibition assays of cell cultures challenged with nitrite

Overnight cultures were diluted to a starting concentration of OD₆₀₀ = 0.02 in YEPD cultured in 13x100 mm culture tubes. The pH of YEPD was adjusted to 3.5, 6.05 or 7.2 by adding HCl or NaOH. Cultures, pretreated with NaCl (control), nitrate or thiocyanate, were incubated for 4 hours in a tissue culture rotator. Nitrite (0-30 mM) was then added to each culture tube. Culture tubes were incubated for an additional 3 hours and the final cell density for each tube was determined by measuring absorbance at 600 nm using a Spec20. Data was normalized for each condition by setting 0 mM nitrite

treated cell cultures equal to 1. For each condition three separate cultures were analyzed and each experiment was repeated three times.

2.6 Tandem affinity purification (TAP) of Cta4 and associated proteins

Construction of TAP tag: The TAP tag was constructed from a Clp20 integration plasmid (Dennison et al., 2005). The *CTA4* putative regulatory 5' region (~1000 bp upstream of the start codon), the open reading frame and a 350 bp 3'UTR was amplified by PCR from genomic DNA. The PCR product was digested with restriction enzymes SacI and NheI, and ligated into Clp20 replacing the URA3 marker to generate *CTA4-Clp20*.

To facilitate introduction of epitope tags DNA encoding unique restriction sites were introduced at both the N-terminus and C-terminus. Synthetic DNA oligos coding for the 3xFLAG tag (DYKDHDGDYKDHDIDYKDDDK) were annealed by heating and rapid cooling and then ligated at the *CTA4* N-terminus to generate 3xFLAG-*CTA4*. The Protein A (ProtA) coding sequence was amplified from plasmid pTA-ProtA-HIS (Lavoie et al., 2008) and ligated into 3xFLAG-*CTA4* at the *CTA4* C-terminus to generate 3xFLAG-*CTA4*-ProtA. Synthetic oligos coding for a ten amino acid spacer and the tobacco etch virus (TEV) protease cleavage site (ENLYFQG) were ligated between the *CTA4* orf and Protein A to yield *CTA4*-TAP (3xFLAG-*CTA4*-spacer-TEV-ProtA). Other plasmids used for tagging Cta4 were also constructed but lacked Cta4 activity in cells or failed to show specific, high-level Cta4-tag cross reactivity. For brevity these constructs are not described here.

2.6.1 Tandem Affinity Purification

C. albicans strain expressing the *CTA4*-TAP tag (in parallel with an untagged control strain) was grown in 2 L YEPD to a final OD₆₀₀ \approx 1.5. Cells were centrifuged, washed 2x with TBS and resuspended in complete lysis buffer (25 ml HEPES pH 8.0, 10% glycerol, 150 mM NaCl, 0.1% NP-40, 1 mM DTT, 1 mM EDTA, 2 mM PMSF, 100 μ M leupeptin, 4 mM benzamidine, 1 μ M pepstatin A, AEBSF 1 mM). In eight separate 2-ml screw cap microfuge tubes, 1 ml of complete lysis buffer was added to 400 mg wet cell pellet along with 0.5-0.7 ml of zirconia/silica beads (Biospec). Cells were lysed by beadbeating in a Mini Beadbeater-8 for five cycles of two minutes at max power, followed by 2 minutes on ice. Lysate was cleared of cell debris by centrifugation at 16,000 *g*, at 4°C for 30 minutes.

Supernatant was collected in a 15 ml centrifuge tube and incubated with 600 μ l packed IgG Sepharose 6 Fast Flow beads (GE Healthcare). Lysate and beads were incubated with gentle rotation at 4°C for 4 hours. Beads were washed with ten column volumes of complete lysis buffer and transferred to a disposable 4 ml Poly-Prep Column (Bio-Rad) and then incubated by gentle rotation at 4°C overnight in 2 ml complete lysis buffer with 15 μ l TEV protease (Promega). The next day TEV cleavage products were separated from IgG beads by gravity flow. Cleavage products were incubated with 50 μ l EZ View Red ANTI-FLAG M2 affinity gel beads (Sigma) in 1.7 ml microfuge tube for 1-2 hours at 4°C with gentle agitation. Beads were washed three times in complete lysis buffer by adding 1 ml buffer to beads followed by 30 second centrifugation at 8200 *g* and aspirating off supernatant. Proteins bound to the ANTI-FLAG affinity gel beads were

eluted by incubating beads with 100 μ l complete lysis buffer with 300 ng/ μ l FLAG peptide (Sigma) for 30 minutes.

2.6.2 Identification of Cta4 interacting proteins by mass spectrometry

Proteins were separated by SDS-polyacrylamide gel electrophoresis and visualized by Coomassie staining. Protein bands of interest were cut out of the gel and placed in a screw cap microfuge tube. The gel slices were washed three times in MilliQ water and dried overnight at room temperature. Samples were sent to Dr. Dick Winant at the Stanford Mass Spectrometry Facility for protein identification by tandem mass spectrometry.

2.7 Chromatin immunoprecipitation (ChIP) assay of Cta4_{9xMyc} binding to the NORE in vivo

Chromatin immunoprecipitation (ChIP) was done according to the protocol described by Aparicio et al. (Aparicio et al., 2005) with the following modifications. Overnight cultures of *C. albicans* strains, wild-type (SN152) and Cta4_{9xMyc}, were diluted to OD₆₀₀ = 0.2 and grown in rich yeast media to a final OD₆₀₀ \approx 1.0. Cell cultures were treated with 10 mM NaNO₂ or 10 mM NaCl (control) and incubated 15 minutes. Cell cultures were then treated with 1% formaldehyde for 20 min. 7.5 ml of 2.5 M glycine was added to the cultures for 5 minutes to quench the formaldehyde. Cells were lysed by beadbeating in a Mini Beadbeater-8 for five cycles of one minute at max power, followed by 2 minutes on ice in 1 ml of lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, and 0.1% SDS) with 2 mM PSMF. Chromatin was sonicated for 3 cycles of 30 sec. at 20 percent power using a Misonix

Sonicator 3000 with Cup Horn to shear chromatin and generate ~500 bp DNA fragments as checked by agarose gel electrophoresis. A 100 µl input sample was removed. The remaining sample (900 µl) was used for immunoprecipitation. Samples were incubated with 50 µl of washed protein G resin (GenScript) and 20 µl of c-Myc (9E10) antibody (SantaCruz) for 1.5 hr at 4°. Protein G resin was washed 3 times in cold lysis buffer and proteins were eluted off the resin by adding 100 µl of elution buffer (50 mM Tris, pH 7.5, 10 mM EDTA, 1% SDS) to resin and incubating in a 65°C water-bath for 10 min to reverse cross-links. The eluate was treated with Proteinase K and incubated for 6 hr at 65°C. DNA was purified by phenol chloroform extraction. PCR was performed using 2 µl of 1/10 dilution of the immunoprecipitate (IP) DNA and 1/15 dilution of input DNA, respectively, in a 50 µl reaction. Products obtained by this PCR program – 2 min initial denature at 94°C, and 26 cycles of 30 sec. denature at 94°C, 30 sec. annealing at 50°C and 1 min. extension at 72°C, 4 min. final extension at 72°C – were analyzed on a 2.5% agarose gel using 5 µl of the input PCR product and 22 µl of anti-Myc immunoprecipitated PCR product.

2.8 In vivo Cta4 co-immunoprecipitation

2.8.1 CTA4-9xMyc, CTA4-6xHA strain construction

A *C. albicans* strain expressing a Myc_{9x} epitope-tagged Cta4 has been described previously (Chiranand et al., 2008). A 6xHA Cta4 epitope tag was generated from the plasmid pSN69-3utrACT1-6HA that contains the 6xHA tag followed by the *CaACT1* 3'UTR followed by the *ARG4* gene from *C. dubliniensis* as a selectable gene for transformation (Noble and Johnson, 2005). A 300 bp fragment of *CTA4* just upstream

the stop codon was amplified and ligated into the pSN69 plasmid just before the 6xHA tag. A second 300 bp fragment of *CTA4* just downstream of the stop codon was amplified and ligated just after the *ARG4* selectable marker. The final product was linearized using restriction enzymes and integrated at the *CTA4* locus by homologous recombination.

2.8.2 Co-immunoprecipitation assay

A *C. albicans* strain in which each copy of Cta4 was tagged at the C-terminus with either 6xHA or 9xMyc (*CTA4*-_{9xMyc}/*CTA4*-_{6xHA}) was grown in 100 ml YEPD to an OD₆₀₀ ~ 0.8. Cell cultures were centrifuged for three minutes at 2000 g. Pellets were washed with TBS, and then resuspended in 1 ml complete lysis buffer with Zirconia/Silica beads in a 2 ml screw cap microfuge tube. Cells were lysed by beadbeating five times for one minute and iced for one minute between cycles. Cell debris was removed by centrifugation at 16,000 g for fifteen minutes at 4°C. 333 µl of cleared supernatant and 5 µl 9E10 anti-myc antibody (Santa Cruz Biotechnology) were incubated by gentle rotation for 2 hours at 4°C. After two hours, 20 µl of Protein G beads (Pierce) was added and incubated with lysate and antibody at 4°C overnight. Samples were washed three times in complete lysis buffer. Proteins were removed from the beads by boiling samples for two minutes in 50 µl 2x sample buffer + β-mercaptoethanol. Samples were centrifuged at top speed for five minutes and 10 µl was loaded onto a SDS-polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane, and non-specific binding was blocked by incubating the membrane in 5% solution dry milk in TBS + 0.1% Tween (TBS-T). The blocked membrane was then probed with 2µl of anti-HA antibody (Rockland) in 10 ml TBS-T with 5% non-fat dry milk for one hour at room

temperature, with gentle shaking. The membrane was washed three times for 5 minutes with TBS-T and then probed with 10 µl of anti-rabbit IgG, HRP-linked antibody (Cell Signaling) in TBS-T with 5% non-fat dry milk for one hour at room temperature. The membrane was washed three times for five minutes with TBS-T. ECL substrate (Pierce) was added to the washed membrane according to the manufactures instructions. Membranes were exposed to film and developed.

2.9 Heterologous expression of Cta4 in *S. cerevisiae* with a P_{CaYHB1} -*lacZ* reporter gene

A 500 bp fragment of the *C. albicans* *YHB1* 5'UTR including the NORE was PCR amplified and ligated into the *S. cerevisiae* centromeric plasmid pSEYC102 (do Valle Matta et al., 2001) to generate a pSEYC102- P_{CaYHB1} -*lacZ* in which the *C. albicans* *YHB1* promoter is driving the *lacZ* reporter gene. A codon optimized version of the *CTA4* open reading frame (sequence in Appendix C), in which all CTG codons were replaced with serine codons, was ligated into plasmid pCM185 (Garí et al., 1997) to generate pCM185-*CTA4*. DNA encoding the 9xMyc tag was inserted before the *CTA4* stop codon to generate pCM185-*CTA4*_{9xMyc}. The pCM185-*CTA4*_{9xMyc} plasmid was transformed into a *S. cerevisiae* strain carrying the pSEYC102- P_{CaYHB1} -*lacZ* reporter gene. β -galactosidase activity was determined according to the protocol described in section 2.3.1.

Chapter 3: *Candida albicans* treated with either DPTA NONOate, nitrite, nitrate or thiocyanate produce a highly similar transcriptional response

3.1 *C. albicans* *YHB1* gene is strongly induced by nitrate

The *C. albicans* flavohemoglobin gene is strongly induced by gaseous nitric oxide and the nitric oxide releasing molecules NOC-18, DPTA NONOate, and nitrite (Hromatka et al., 2005; Ullmann et al., 2004). The *YHB1* gene product consumes nitric oxide in an oxygen dependent manner to generate nitrate. This activity is predicted to protect *C. albicans* against the toxic effects of nitric oxide generated by inducible nitric oxide synthases (iNOS) in phagocytic cells. In addition to nitric oxide generated by iNOS, nitrite generated by commensal bacteria in the mouth (Weitzberg and Lundberg, 1998), gut (Sobko et al., 2005) and skin (Weller et al., 1996), has also been shown to inhibit microbial growth. Nitrite production by commensal bacteria is thus thought to play an integral part of innate host defense against invading pathogens (Lundberg et al., 2004).

To test whether nitrite generated from nitrate by a commensal oral bacterium is sufficient to induce expression of a *YHB1-lacZ* reporter gene in *C. albicans*, a former member of the lab (Erin Stashi) co-cultured *C. albicans* expressing a *YHB1-lacZ* reporter gene with the oral bacterium *Rothia mucilaginosa*, a constituent of the normal human oral microbial flora. Nitrate was added to the co-cultures to allow the generation of nitrite by *Rothia mucilaginosa* via nitrate reductase activity. For one of the negative control conditions, *C. albicans* with nitrate but without *R. mucilaginosa*, the *YHB1-lacZ* reporter gene was strongly induced. This result was surprising for different reasons. Nitrate is the

product of nitric oxide detoxification and is nontoxic to cells. Also, unlike many filamentous fungi and bacteria, *C. albicans* cannot utilize nitrate as a nitrogen source for growth (Pincus et al., 1988; data not shown) and the addition of nitrate to media does not improve growth of *C. albicans* under anaerobic conditions (Dumitru et al., 2004). The latter results are consistent with *C. albicans* being unable to use nitrate as a terminal electron acceptor during anaerobic growth. The *C. albicans* database (www.candidagenome.org) does not have an annotation for a nitrate transporter or nitrate reductase, and BLAST results using nitrate transporter and reductase sequences from both bacterial and fungal species does not generate any BLAST hits with significant sequence homology.

Based on the preliminary data, I measured *YHB1-lacZ* expression in response to increasing amounts of nitrite or nitrate by β -galactosidase assay. Cells expressing the reporter gene were grown in rich yeast media (YEPD) at pH 6.0 to mid-log phase and treated with increasing amounts of nitrite or nitrate. As shown in Figure 3-1, β -galactosidase activity shows strong induction of *YHB1-lacZ* expression in response to nitrite or nitrate treatment over a similar concentration range.

Induction of flavohemoglobin in response to nitrate is seen in bacteria and fungi that are able to reduce nitrate for respiration or assimilation (Crack et al., 2008; Schinko et al., 2010). This response to nitrate under nitrate assimilation conditions is presumed to be the result of nitric oxide generated as an intermediate during the reduction of nitrate to nitrous oxide. As *C. albicans* has no known orthologs of any of the nitrate utilizing genes, we wanted to know if nitrate could be acting as the signal that induces *YHB1* expression.

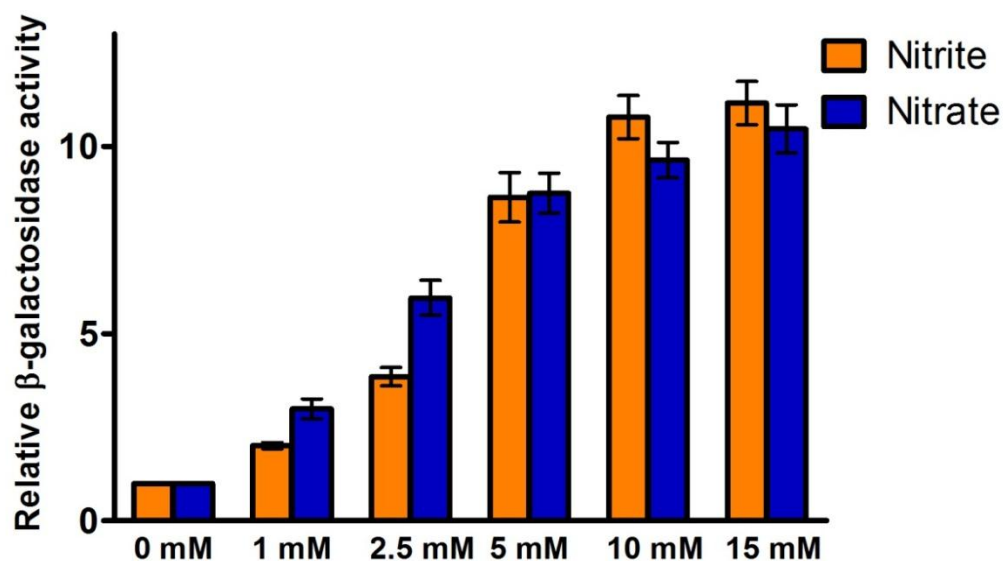


Figure 3-1. *YHB1-lacZ* reporter gene expression in *C. albicans* is induced in response to nitrite or nitrate

A 16xNORE-*YHB1-lacZ* fusion reporter gene was transformed into a *C. albicans* wild-type strain. This reporter strain was grown in rich yeast media (YEPD) buffered to pH 6 and treated with increasing amounts of nitrite or nitrate. *YHB1-lacZ* expression levels were determined by measuring β -galactosidase activity. Data were normalized between each experiment by expressing relative β -galactosidase activity as the fold increase in activity above untreated control (untreated: 0 mM). Results are from three separate experiments. Error bars = ± 1 S.E.M.

In *C. albicans* there is some basal nitric oxide consumption activity in cells that is *YHB1*-, but not *YHB4*- or *YHB5*-dependent (Ullmann et al., 2004). One question I wanted to address is whether *YHB1* is induced in the absence of a functional Yhb1 protein, i.e., is the conversion of nitric oxide to nitrate necessary for *YHB1* induction, or is nitric oxide able to induce expression of *YHB1*? To address this question, I transformed the *lacZ* reporter gene under control of the *YHB1* promoter (*P_{YHB1}-lacZ*) lacking the *YHB1* open reading frame, into a *yhb1Δ/Δ* deletion strain. Induction of *lacZ* expression, determined by β -galactosidase assay, was used as a measure of *YHB1* transcriptional activation. The results in Figure 3-2 show that induction of *YHB1* by nitrite (nitric oxide) is not dependent on Yhb1 protein function. This data shows that *C. albicans* has the ability to sense and respond to both nitrite and nitrate. Another implication of this result is that flavohemoglobin is apparently not required for sensing nitrite, i.e., nitric oxide. *YHB1* promoter activity in response to both nitrite and nitrate is slightly higher in Figure 3-1 compared to Figure 3-2 because we have found that expressing the *lacZ* fused to *YHB1* (*YHB1-lacZ*) results in greater β -galactosidase activity.

An alternative explanation of nitrate induction of *YHB1* is that the mechanism by which *C. albicans* senses nitric oxide and nitrate involves promiscuous binding of the chemically related ligands. To test the selectivity of *YHB1* induction by nitric oxide and nitrate, I measured *YHB1-lacZ* expression in cells treated with other oxyanions (oxygen containing ionic compounds) by β -galactosidase assay. The treatment of *C. albicans* with other oxyanions did not result in induction of the *YHB1-lacZ* reporter gene (Figure 3-3).

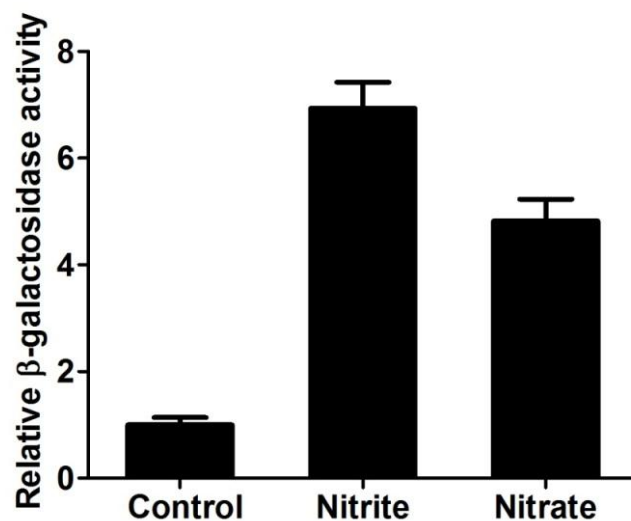


Figure 3-2. *YHB1* promoter activity in a *yhb1 Δ* mutant strain in cells treated with nitrite or nitrate

A reporter gene (*lacZ*) driven by the *C. albicans* *YHB1* promoter was transformed into a *yhb1 Δ* mutant strain. Transformants were treated with 10 mM of nitrite or nitrate (or 10 mM NaCl, control) and promoter activity was determined by measuring β -galactosidase activity. Data were normalized between each experiment by expressing relative β -galactosidase activity as the fold increase in activity above untreated control. Results are from three separate experiments. Error bars = ± 1 std. dev.

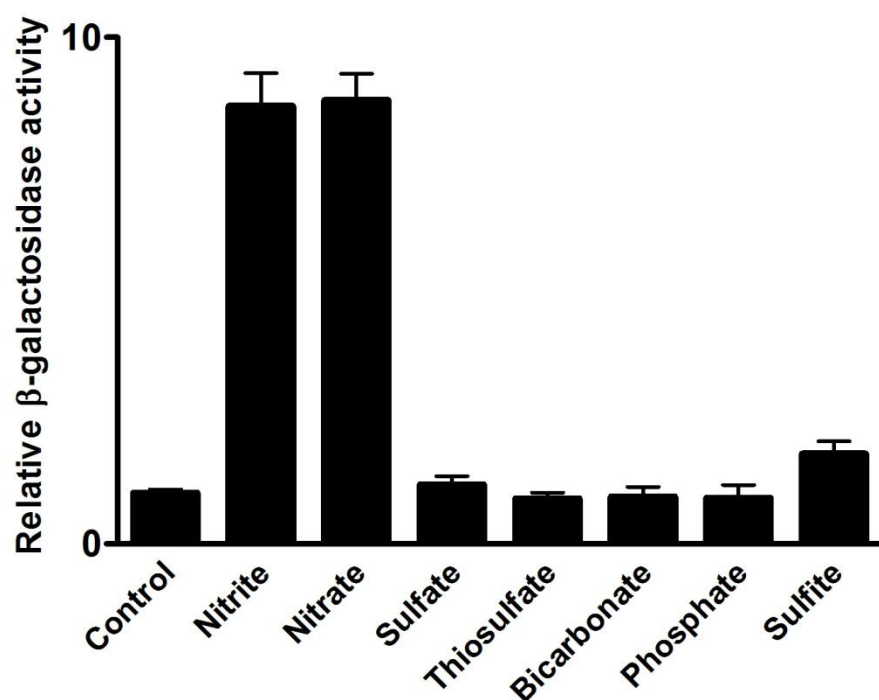


Figure 3-3. Comparison of *YHB1-lacZ* induced expression in response to nitrite, nitrate or other oxyanions

A *YHB1-lacZ* fusion reporter gene was transformed into the *C. albicans* wild-type strain SN152. This reporter strain was grown in rich yeast media and cell cultures were exposed to 5 mM of each oxyanion for one hour. *YHB1-lacZ* expression levels were determined by measuring β -galactosidase activity. Data were normalized between each experiment by expressing relative β -galactosidase activity as the fold increase in activity above untreated control. Results are from three separate experiments. Error bars = ± 1 S.E.M.

3.2 The pseudohalide thiocyanate and the halides iodide and bromide induce expression of a *YHB1-lacZ* reporter gene

As a result of the unexpected finding that nitrate is able to induce *YHB1-lacZ* expression in *C. albicans*, we examined the literature concerning nitrate and other ions in body fluids. *C. albicans* as an obligate commensal organism living on mucosal surfaces of different epithelia would be exposed to nitrate in various secretions including: sweat, bile, and saliva. As much as 25% of all nitrate in saliva is actively transported by salivary glands, resulting in salivary nitrate levels that are tenfold higher than plasma levels (Weitzberg and Lundberg, 1998). The exact mechanism by which nitrate is transported from the blood into saliva is not known. However, the pseudohalide thiocyanate and iodide are also actively transported by salivary glands and these three molecules appear to share the same transport mechanism (Fletcher et al., 1956). To test if this connection between nitrate, thiocyanate and iodide extends to *C. albicans*, I measured the transcriptional response of *YHB1-lacZ* to thiocyanate or different halides (Figure 3-4). Thiocyanate and iodide were able to significantly induce *YHB1-lacZ* expression. Induction by bromide was statistically significant, but only two-fold higher than untreated control. Thus transcriptional activation of the *YHB1-lacZ* gene occurs in response to nitric oxide, nitrate, thiocyanate and iodide, but not to the other tested anions.

Thiocyanate (SCN^-) is oxidized to toxic hypothiocyanite (OSCN^-) by neutrophils in the oral cavity and by peroxidases secreted in saliva, milk, and airway mucus. The ability of peroxidases to catalyze the oxidation of thiocyanate, while consuming H_2O_2 , is an important part of the innate mucosal defense against pathogenic microorganisms

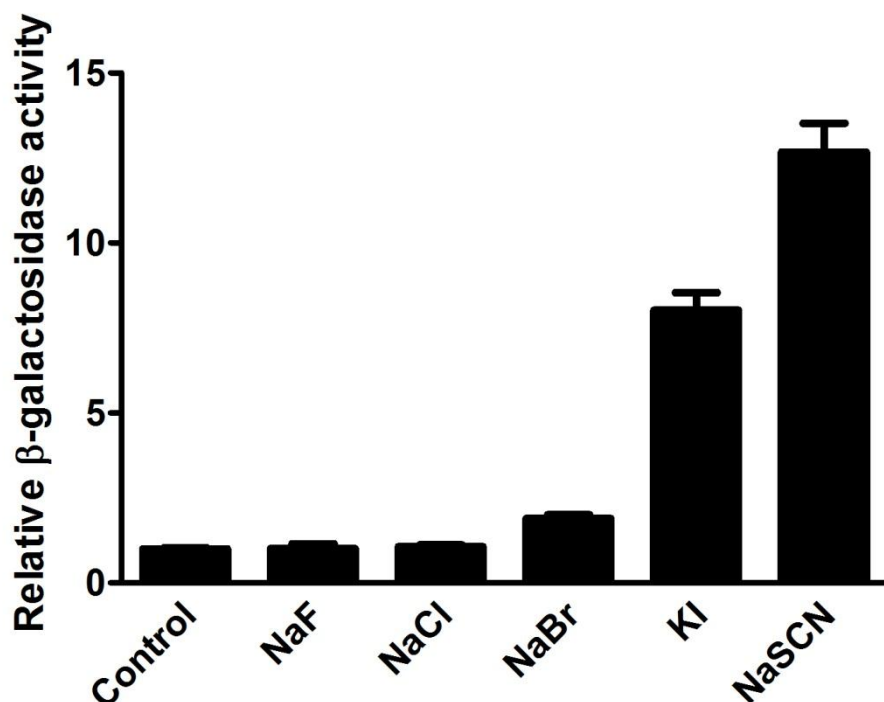


Figure 3-4. Induction of *YHB1-lacZ* expression by halides and the pseudohalide thiocyanate (SCN^-)

A reporter strain carrying the *YHB1-lacZ* reporter gene was grown in rich yeast media (YEPD) and exposed to 5 mM of each halide or pseudohalide for one hour. *YHB1-lacZ* expression levels were determined by measuring β-galactosidase activity. Data were normalized between each experiment by expressing relative β-galactosidase activity as the fold increase in activity above untreated control. Results are from three separate experiments. Error bars = ± 1 S.E.M.

(Ihalin et al., 2006). Certain species of soil bacteria are able to utilize thiocyanate as a sole source of nitrogen (Sorokin et al., 2001) or sole source of sulfur (Stratford et al., 1994).

To determine if *C. albicans* can use thiocyanate as a possible nutrient, a prototrophic strain of *C. albicans* (SC5314) was tested for growth on nitrogen-free medium with or without thiocyanate. Cells showed a similar lack of growth on both media, but grew well on plates that were supplemented with ammonium sulfate as a nitrogen source. Yasmin Chebaro tested the ability of *C. albicans* to utilize thiocyanate as a sulfur source by growing the SC5314 strain in sulfur free liquid media with various sulfur sources including cysteine, methionine, sulfate, sulfite, thiocyanate or no sulfur source. Overnight growth of SC5314 in media with or without sulfur source was determined by measuring the OD₆₀₀ for each culture. Although the other sulfur sources supported growth, thiocyanate did not. The optical density of the cultures grown in thiocyanate as a sulfur source was identical to cultures with no added sulfur source. Together these results are consistent with thiocyanate, rather than a metabolite derived from thiocyanate, directly inducing *YHB1* expression. Based on whole genome array data and reporter gene assay we know that *YHB1* is not induced by oxidative stress (Ullmann et al., 2004), heat stress or osmotic stress (Enjalbert et al., 2003). The transcriptional response of *YHB1* is specifically elicited by nitric oxide, nitrite, nitrate, the pseudohalide thiocyanate or the larger halide iodide. It is unclear why only thiocyanate and iodide strongly induce *YHB1* while bromide, chloride and fluoride induce *YHB1* very little or not at all. It may be that the larger halide iodide and large pseudohalide

thiocyanate bind to the sensor better than the smaller halides bromide, chloride and iodide.

3.3 A similar genome transcriptional response is elicited by DPTA NONOate, nitrite, nitrate or thiocyanate in *C. albicans*

To more completely compare the cellular responses of different *YHB1*-inducing chemicals, we compared the transcript profile of *C. albicans* in response to DPTA NONOate, nitrite, nitrate or thiocyanate. Nitrite is non-enzymatically converted to nitric oxide at acidic pH. In our lab we have found 5 mM nitrite at pH 6.0 is sub-toxic and is able to induce *YHB1-lacZ* expression. DPTA NONOate is a pH-dependent nitric oxide donor that releases two moles of nitric oxide for every mole of compound. To ensure all cells were grown under identical conditions (same media and same pH) for all microarray experiments, I used a concentration of DPTA NONOate (0.02 mM) that is sub-toxic and induces comparable *YHB1-lacZ* expression levels at pH 6 compared to 5 mM nitrite. I used 5 mM nitrate or thiocyanate (or NaCl as a control) based on the ability of these compounds to induce *YHB1-lacZ*. Thiocyanate was used and not iodide because even though both thiocyanate and iodide are concentrated in the saliva 20-30 fold above plasma concentrations (Fite et al., 2004), the concentration of thiocyanate in saliva is 10-100 fold greater than either iodide or bromide (Xu et al., 2008), making it more physiologically relevant at the concentrations necessary to induce *YHB1*.

To compare transcript profile in response to these compounds the laboratory strain SN152 was grown to mid-log phase ($OD_{600} = 1.0$) in YEPD at pH 6 in a shaker (200 rpm) at 30°C. The culture was then split into equal volumes and treated for ten

minutes with DPTA NONOate, NaNO₂, NaNO₃, NaSCN, or NaCl as a control, at the concentrations indicated above. Total RNA was extracted and RNA quality and concentration was determined using a NanoDrop 1000 spectrophotometer. RNA samples were submitted to the Microarray Core Laboratory at the University of Texas Health Science Center at Houston for cDNA synthesis, labeling, and chip hybridization (see *Materials and Methods*). For each condition two biological and two technical (dye-swap) replicates were analyzed and expressed as fold changes in expression relative to control. Only genes with *P* values <0.05 for all conditions are shown in the heat map in Figure 3-5. I generated a second heat map by converting all expression ratios with *P* values <0.05 to log base 2 transformed values (Appendix D, Figure S1). Using cut-off of 2 I identified only 24 genes that were significantly induced or repressed under any of the four tested conditions. All but 3 of the genes were upregulated under these conditions with *YHB1*, *SSU1* and *orf19.3120* being the only genes significantly induced under all conditions.

Each of the four conditions induced a similar, small set of genes. Absolute induction ratio values are listed in Table 1. For each of the conditions tested, *YHB1* was the most highly upregulated of all genes. One of the other highly upregulated genes was

Table 1. Absolute expression ratios of genes induced with *P* values <0.05 for all conditions

Gene name	DPTA NONOate	Nitrite	Nitrate	Thiocyanate
<i>YHB1</i>	196.1	47.6	70.7	84.1
<i>SSU1</i>	14.5	5.1	8.2	6.1
<i>orf19.3120</i>	9.3	6.0	9.0	6.8
<i>RHR2</i>	3.2	2.3	1.5	6.1
<i>CTR2</i>	4.2	2.0	2.2	2.7
<i>SGA1</i>	2.6	1.6	2.9	2.3
<i>CTA4</i>	2.3	2.3	2.1	2.3

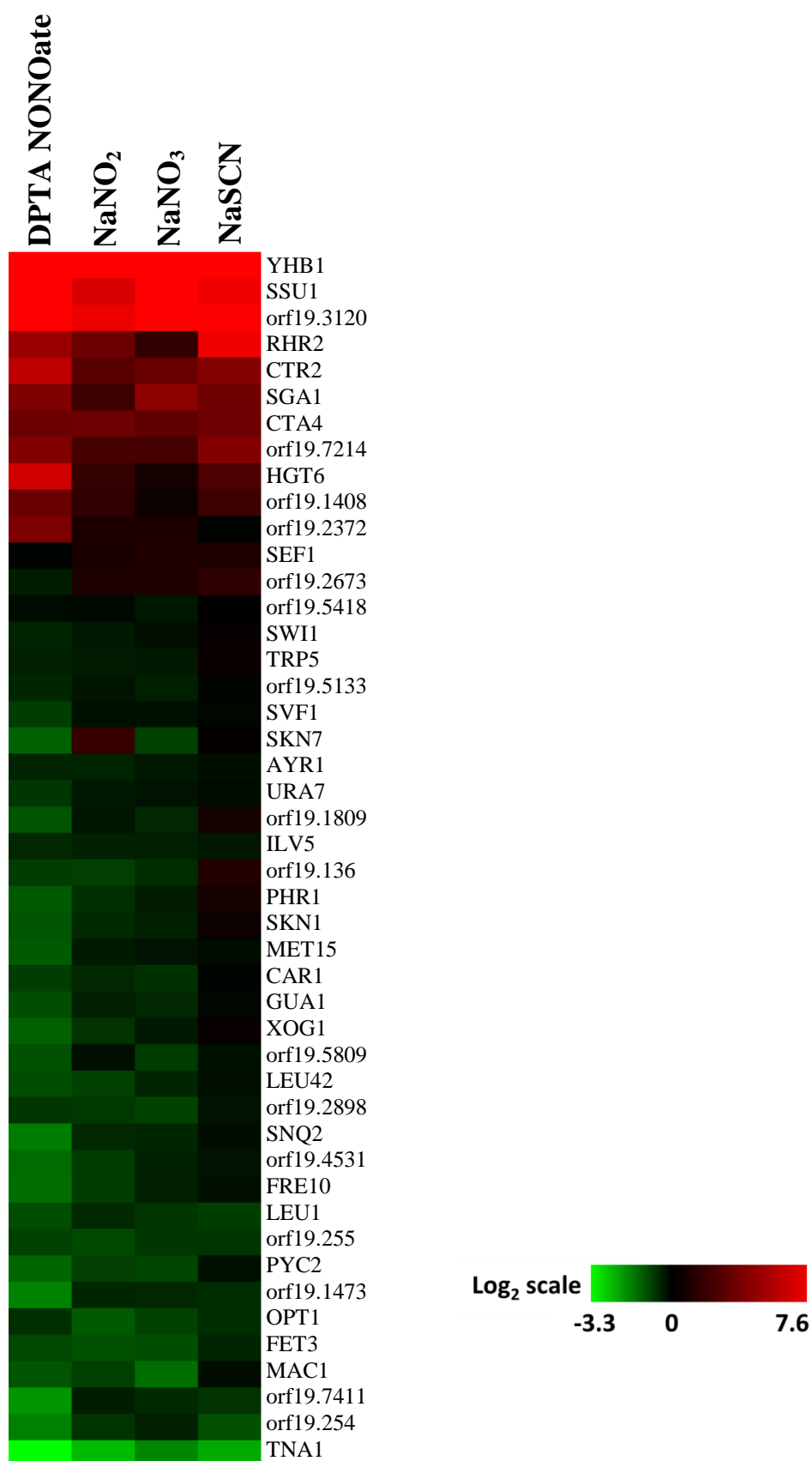


Figure 3-5. Genome-wide expression profile of *C. albicans* in response to nitric oxide, nitrite, nitrate or thiocyanate

Log-phase wild-type *C. albicans* cells (strain SN152) grown in rich yeast media, pH 6, were treated for 10 minutes with 5 mM NaNO₂, 5 mM NaNO₃, 5 mM, 0.02 mM DPTA NONOate or 5 mM NaCl (control). All expression values, from averages of two biological and technical replicates, are represented as the ratio of expression levels in treated versus control sample. Ratio values were converted to log base 2 and visualized using Treeview (Eisen et al., 1998). In this “heat map” red indicates induction and green indicates repression. Only genes with *P* values <0.05 for all four conditions tested are shown.

SSU1. *SSU1* encodes a putative sulfite transporter; a mutant lacking *SSU1* is sensitive to exogenous sulfite in both *C. albicans* (Chiranand et al., 2008) and *S. cerevisiae* (Park and Bakalinsky, 2000). In both *C. albicans* (Hromatka et al., 2005) and *S. cerevisiae* (Sarver and DeRisi, 2005), *SSU1* is strongly induced by nitric oxide, however deletion of this gene has no effect on nitric oxide sensitivity in either *C. albicans* or *S. cerevisiae* when grown in YEPD. In *S. cerevisiae* the transcription factor Fzf1p regulates both *SSU1* and *YHB1* expression (Sarver and DeRisi, 2005). In *C. albicans*, *SSU1* induction by nitric oxide is partially *CTA4* dependent (Chiranand et al., 2008). Alice Fa in the Gustin lab has shown that nitrate pretreatment induces a *CTA4*-dependent increase in sulfite resistance in *C. albicans* (data not shown).

The other highly induced gene for all conditions is the open reading frame *orf19.3120*. This gene is annotated as one of 26 putative PDR-subfamily ABC transporter genes in the *Candida* Genome Database (CGD) (Braun et al., 2005). To determine if this gene has a role in nitric oxide resistance or *YHB1* induction, a strain was constructed by Nicole Marino, a former lab member, in which both copies of the open reading frame were deleted. I measured *YHB1* expression by qRT-PCR in response to nitrite, nitrate and thiocyanate using this *orf19.3120Δ/Δ* mutant strain. In this strain *YHB1* is expressed at wild-type levels under all tested conditions and deletion of *orf19.3120* does not result in increased sensitivity to nitrite. Other strongly induced genes are the glycerol 3-phosphatase *RHR2* and the putative low-affinity vacuolar membrane copper transporter *CTR2*. I measured *YHB1* mRNA levels in response to nitrite, nitrate and thiocyanate by qRT-PCR in *rhr2Δ/Δ* (Bonhomme et al., 2011) and *ctr2Δ/Δ* (Noble et al., 2010) deletion strains. In both strains *YHB1* was induced at, or

near, wild-type levels under all tested conditions and neither deletion strain showed increased sensitivity to nitrite compared to wild-type (data not shown). *RHR2* is induced under osmotic and oxidative stress conditions (Smith et al., 2004) and deletion of this gene leads to reduced salt tolerance (Fan et al., 2005), but I found that *RHR2* is not necessary for full nitrite stress resistance (data not shown). Similarly, *SSU1*, *CTR2* and *orf19.3120* are not necessary for nitrite resistance. It is unclear why these genes are induced under all conditions. Of all significantly induced genes, only *YHB1* and *CTA4* have been shown to be necessary to protect against nitric oxide inhibition of growth.

Microarray analysis identified both *YHB1* and *CTA4* as genes significantly induced under all tested conditions. I was unable to identify additional genes necessary for *YHB1* regulation or nitrite resistance based on my microarray analysis. I attempted to identify any additional genes that may have a role specifically in the nitric oxide stress response. To do this I identified three fungal specific, uncharacterized genes that are significantly induced by both DPTA NONOate and nitrite: *orf19.7091*, *orf19.5785* and *orf19.8119*. To further characterize these genes I deleted both copies of the open reading frame using the lab strain SN152 with a *YHB1-lacZ* reporter gene as the parent strain. Each deletion strain was tested for sensitivity to nitrite by growth assay and for the ability to induce the *YHB1-lacZ* reporter gene in response to nitrite, nitrate or thiocyanate. All three deletion strains showed wild-type levels of nitrite resistance and *YHB1-lacZ* induction (data not shown).

Chapter 4: Comparison of flavohemoglobin gene expression in *C. albicans*, *S. cerevisiae* and *Aspergillus nidulans* in response to nitrite, nitrate and thiocyanate

A similar transcriptional response of *C. albicans* to DPTA NONOate and nitrite was predicted from previous work. The upregulation of flavohemoglobins in response to these nitric oxide donors is nearly ubiquitous in microorganisms; however, the transcriptional response of *C. albicans* to both nitrate and thiocyanate is a novel finding. In both *E. coli* and *Aspergillus nidulans*, organisms able to reduce nitrate, induction of the flavohemoglobin gene is presumed to be in response to the formation of nitric oxide as a by-product of nitrate reduction. As *C. albicans* is unable to reduce nitrate and lacks orthologs to nitrate transport and nitrate reductase genes, I wanted to see if this response to nitrate and thiocyanate is conserved in the model yeast *Saccharomyces cerevisiae* and the filamentous fungi *Aspergillus nidulans*.

S. cerevisiae is estimated to have diverged from *C. albicans* 140-800 million years ago (Berman and Sudbery, 2002). Although there is a high degree of homology in gene sequence and function between the two species, 1,218 unique genes in the *Candida* Genome Database are identified as having no significant homolog in any database (Braun et al., 2005) and there is significant rewiring of transcriptional circuits between the two yeast (Homann et al., 2009). The differences between these two organisms are thought to support survival and growth in the different niches each of these organisms occupy. Like *C. albicans*, *S. cerevisiae* is unable to utilize nitrate as a sole nitrogen source (Walker, 1998).

To determine if nitrate or thiocyanate is able to induce the *S. cerevisiae* *YHB1* gene (*ScYHB1*) I constructed a plasmid in which 900 bp upstream of the *ScYHB1* open reading frame was amplified and this putative promoter region was fused in frame to a *lacZ* gene reporter gene. I transformed this plasmid into the *S. cerevisiae* laboratory strain BY4742. Figure 4-1 shows the *ScYHB1* promoter activity measured by β -galactosidase assay in response to nitrite, nitrate, thiocyanate or control (NaCl). The induction of the *lacZ* gene was nearly 10 fold higher in response to nitrite compared to control. However, in response to both nitrate and thiocyanate treatment there was no significant change in *ScYHB1* promoter activity.

Unlike *YHB1* in *S. cerevisiae*, the flavohemoglobin gene (*fhbA*) in *Aspergillus nidulans* (*fhbA*) is induced in response to not only nitric oxide and nitrite, but also to nitrate (Schinko et al., 2010). To determine whether thiocyanate induces expression of *fhbA* I measured its mRNA transcript levels by qRT-PCR. The flavohemoglobin gene, *fhbA*, was strongly induced under all previously tested conditions (Figure 4-2). In response to thiocyanate *fhbA* was induced three-fold above non-treated control (Figure 4-2). While modest compared to DETA NONOate, nitrite and nitrate, this result indicates that *A. nidulans* is able to sense and respond to thiocyanate by inducing expression of a flavohemoglobin gene.

Aspergillus nidulans was tested for the ability to utilize thiocyanate as a sole source of nitrogen or sulfur by measuring growth on agar plates. On glucose minimal media plates, *A. nidulans* was not able to grow on plates supplemented with thiocyanate as a nitrogen source. However, *A. nidulans* was able to grow on glucose minimal media plates with thiocyanate as the sole source of sulfur (communication, Dr. Greg May). The

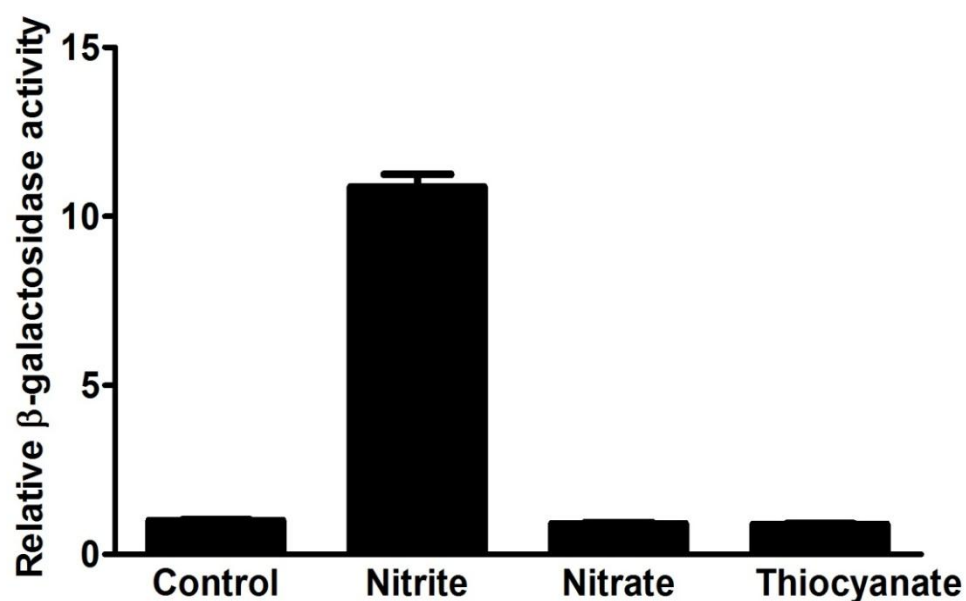


Figure 4-1. The *Saccharomyces cerevisiae* *YHB1* promoter is activated by nitrite but not nitrate or thiocyanate

A centromeric plasmid (pSEYC102) with the *S. cerevisiae* *YHB1* promoter fused to the *lacZ* open reading frame was transformed into *S. cerevisiae* strain BY4742. *lacZ* expression levels were determined by measuring β -galactosidase activity of cultures treated with nitrite, nitrate or thiocyanate. Data were normalized between each experiment by expressing relative β -galactosidase activity as the fold increase in expression above mock-treated control (NaCl). Results are from three separate experiments. Error bars = ± 1 S.E.M.

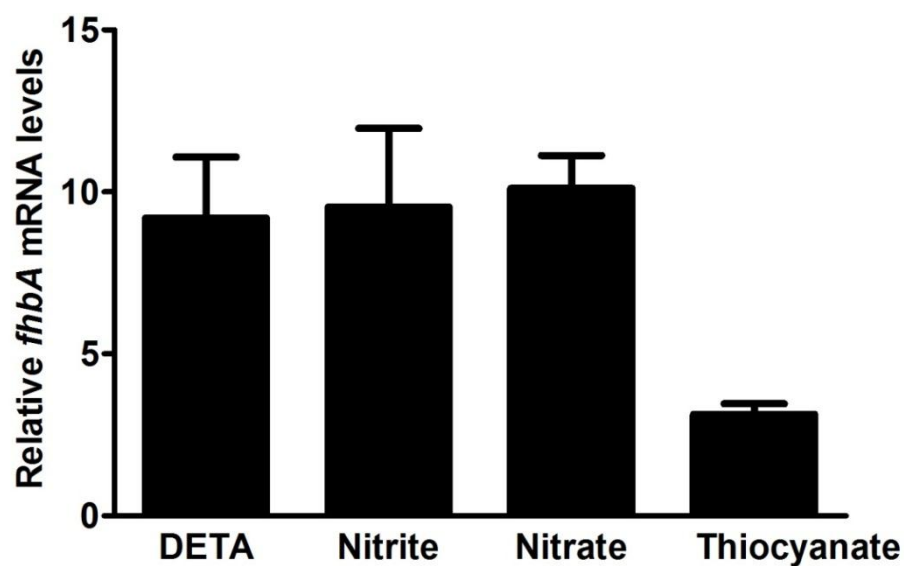


Figure 4-2. The *Aspergillus nidulans* flavohemoglobin gene (*fhbA*) is induced by nitric oxide (DETA NONOate), nitrite, nitrate and thiocyanate *A. nidulans* strain R21 was grown in nitrogen free media and treated with 3 mM DETA (NONOate), 10 mM nitrite, nitrate, thiocyanate or NaCl (control) as previously described (Schinko et al., 2010). The relative *fhbA* mRNA levels are expressed as fold increase in expression relative to the control. Results are from three separate experiments. Error bars = ± 1 S.E.M.

ability of *A. nidulans* to utilize thiocyanate as a sulfur source and not a nitrogen source is surprising because ammonia is formed in both proposed mechanisms of thiocyanate biodegradation. In the carbonyl pathway, the enzyme thiocyanate hydrolase catalyzes the conversion of thiocyanate to ammonia and carbonyl sulfide ($\text{C}=\text{O}=\text{S}$), with carbonyl sulfide eventually being hydrolyzed to carbon dioxide and hydrogen sulfide, which is eventually oxidized to sulfate. In the cyanate pathway thiocyanate is hydrolyzed to hydrogen sulfide (HS^-) plus cyanate (CNO^-) which is converted to ammonia and carbon dioxide by the enzyme cyanase (Ebbs, 2004). Cyanase is found in bacteria, fungi and plants and the *A. nidulans* genome database has a gene (AN7331) computationally annotated as having a role in cyanate metabolism (www.aspergillusgenome.org). Because *A. nidulans* is not able to utilize thiocyanate as a nitrogen source, it seems unlikely that the flavohemoglobin expression in response to thiocyanate is a result of nitric oxide formation during the reduction of the nitrogen compounds generated during the utilization of thiocyanate. What is still unknown is whether thiocyanate shares the same sensing and signaling pathway with nitrate in either *A. nidulans* or *C. albicans*.

Chapter 5: Induction of *YHB1* by nitrite, nitrate and thiocyanate is *CTA4* dependent, and stronger at acidic pH

5.1 Induction of *YHB1* by nitrite is pH dependent

Both nitrite and nitrate at pH 6 are able to activate transcription of a *lacZ* reporter gene when placed under control of the *YHB1* promoter in a strain lacking functional Yhb1 protein (*yhb1Δ/Δ*). In that study nitrite was used as a nitric oxide source assuming that nitric oxide is generated at acidic pH by the non-enzymatic breakdown of nitrite. However, in light of our findings that nitrate is able to induce *YHB1*, and that the *A. nidulans* flavohemoglobin gene is induced by nitric oxide, nitrate and nitrite (at pH 6.8) (Schinko et al., 2010), it was important to determine whether the conversion of nitrite to nitric oxide at acidic pH is necessary for *YHB1* induction or if nitrite alone is sufficient to induce transcription. To distinguish between these possibilities *YHB1* mRNA levels were measured in cells treated with nitrite, nitrate or thiocyanate at pH 5.0 or pH 7.2. At pH 7.2, nitrite is relatively stable and its conversion to nitric oxide is therefore minimal. For this experiment overnight cultures of the wild-type strain SN152 were diluted into YEPD media adjusted to pH 5 or pH 7.2 and log phase cultures ($OD_{600} = 1.0$) were treated with either 5 mM $NaNO_2$, 5 mM $NaNO_3$, 5 mM $NaSCN$ or 5 mM $NaCl$ (control) for 10 minutes and *YHB1* transcript levels were analyzed by quantitative RT-PCR.

For nitrite-treated cells the transcript levels of *YHB1* mRNA were significantly lower at pH 7.2 compared to pH 5.0 (Figure 5-1). In contrast, both nitrate and thiocyanate were able to induce *YHB1* mRNA transcript levels to high levels at both pH levels tested.

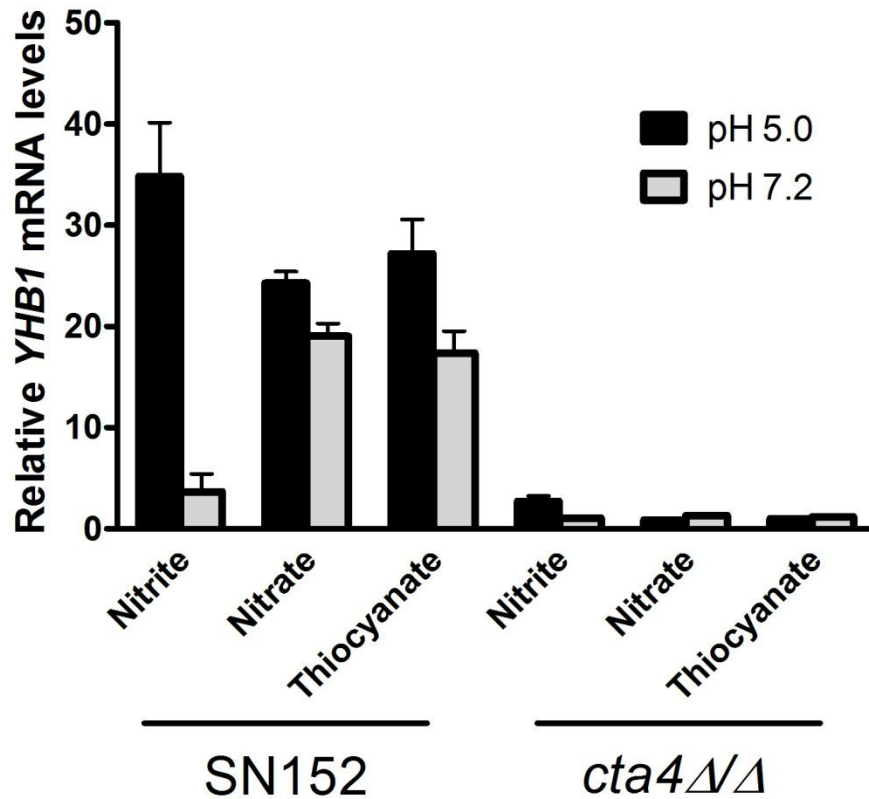


Figure 5-1. *YHB1* mRNA transcript levels in *C. albicans* wild-type strain SN152 and the deletion mutant *cta4Δ/Δ* at pH 5.0 and pH 7.2

Each strain was grown in rich yeast media (YEPD) at pH 5.0 or 7.2, and treated with 5 mM of NaNO₂, NaNO₃, NaSCN or NaCl (control) for 10 minutes.

Relative *YHB1* mRNA levels are expressed as fold increase in expression relative to untreated control. Results are from three separate experiments. Error bars = ± 1 S.E.M.

These results support the hypothesis that the breakdown of nitrite to nitric oxide at acidic pH is necessary for *YHB1* induction, however, these results do not necessarily exclude the possibility that other products of nitrite breakdown at acidic pH are responsible for *YHB1* induction.

Figure 5.1 shows the increase in *YHB1* mRNA levels after a 10 minute exposure to nitrite, nitrate or thiocyanate. To determine whether the presence of nitrate and thiocyanate are able to maintain elevated *YHB1* expression levels by continually activating transcription, I compared relative *YHB1* mRNA levels of cells exposed to nitrate or thiocyanate for 10 minutes, two hours, and 4 hours. Cells that were exposed to nitrate or thiocyanate for 4 hours show equivalent *YHB1* mRNA expression levels compared to cells exposed for only 10 minutes (data not shown). This indicates that there is no obvious desensitization to nitrate or thiocyanate and as long as those compounds are present, *YHB1* transcription is apparently sustained at elevated levels, under these growth conditions. Hromatka et al. showed similar results in which *YHB1* was persistently induced when treated with DPTA NONOate (Hromatka et al., 2005).

5.2 Induction of *YHB1* by nitrate and thiocyanate is *CTA4* dependent

In nitrate reducing organisms such as *E. coli* and *A. nidulans*, expression of flavohemoglobin genes by nitrate has been attributed to the presence of nitrate reduction products, specifically nitric oxide (Zumft, 1997). In *C. albicans* the transcriptional regulator *CTA4* is required for *YHB1* expression in response to nitric oxide or nitrite (Chiranand et al., 2008). To determine whether *CTA4* is similarly necessary for *YHB1*

induction by nitrate or thiocyanate, *YHB1* mRNA levels were measured in a *cta4Δ/Δ* mutant strain treated with nitrate or thiocyanate.

For this experiment overnight cultures of *cta4Δ/Δ* mutant strain were diluted into fresh YEPD media adjusted to pH 5.0 or pH 7.2 and log phase cultures ($OD_{600} = 1.0$) were treated with either 5 mM $NaNO_2$, 5 mM $NaNO_3$, 5 mM $NaSCN$ or 5 mM $NaCl$ (control) for 10 minutes and then analyzed mRNA transcript levels by quantitative RT-PCR. As shown in Figure 5-1, *YHB1* is not induced by nitrate or thiocyanate at either pH 5.0 or pH 7.2 in cells lacking *CTA4*.

5.3 *YHB1* is induced by physiologically relevant concentrations of nitrate and thiocyanate, with greater induction at low pH

Measurements of *YHB1* mRNA levels at pH 5.0 and pH 7.2 show that nitrate and thiocyanate induced higher *YHB1* mRNA transcript levels at the lower pH. Under non-inducing conditions, *YHB1* transcript levels are similar at both pHs. Indeed *YHB1* is not induced by lowering the media pH (Bensen et al., 2004). Unlike nitrite, both nitrate and thiocyanate are stable at acidic pH.

To determine whether environmental pH affects *YHB1* transcript levels when treated with nitrate or thiocyanate, I measured *YHB1* mRNA levels in cells treated with nitrate or thiocyanate at pH 3.5 and pH 6.0. For this experiment, in addition to testing a broader range of physiologically relevant pH levels, I also reduced the levels of nitrate and thiocyanate to levels more consistent with physiological concentrations found in saliva. In normal adult saliva the amount of nitrate can range from 0.5 -2.6 mM (Sádecká and Polonský, 2003) and can even be as high as 10 mM following a meal of nitrate rich

foods (Lundberg et al., 2008). Thiocyanate levels vary less than nitrate levels and range from 0.5 -2 mM (Tsuge et al., 2000).

The data in Figure 5-2 shows treatment of cells with 1 mM nitrate or 1 mM thiocyanate increased the level of *YHBI* transcript levels after only 10 minute exposure to either of these compounds. Consistent with what was observed at pH 5.0 and pH 7.2, an increase in *YHBI* mRNA levels in response to both nitrate and thiocyanate was significantly greater at the lower pH 3.5 compared to pH 6.0.

It is unclear whether the pH-dependent effects of nitrate and thiocyanate on induction of *YHBI* are related to increased uptake of these ligands due to proton dependent membrane transport. This could account for greater intracellular nitrate accumulation resulting in higher levels of *YHBI* expression. In *Arabidopsis*, for example, nitrate enters the cells by a coupled transport in which a proton-nitrate symporter cotransports hydrogen atoms with every nitrate ion (Ho et al., 2009). In *Arabidopsis*, both nitrate and pH are signals that regulate expression of the *CHL1* gene, which codes for a nitrate transporter (Tsay et al., 1993). However it is not known whether nitrate induction of *YHBI* expression requires the translocation of nitrate across the cell membrane.

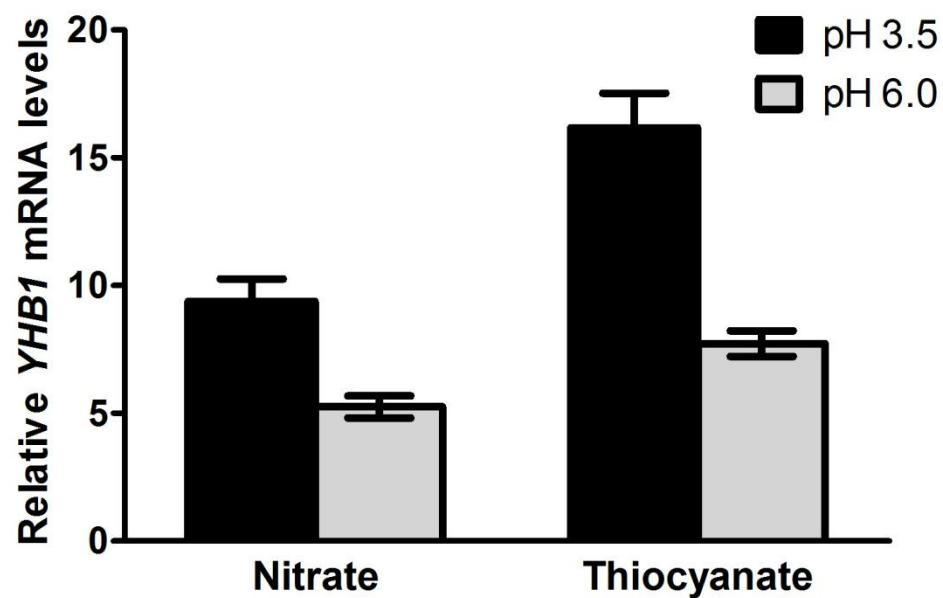


Figure 5-2. Fold induction of the *YHB1* gene by nitrate and thiocyanate is greater at pH 3.5 compared to pH 6.0

C. albicans wild-type strain SN152 was grown in rich yeast media (YEPD) pH adjusted to pH 3.5 or pH 6.0 with HCl and treated with 1 mM of NaNO₃ or NaSCN for 10 minutes. The relative *YHB1* mRNA levels are expressed as fold increase in expression relative to untreated control determined by qRT-PCR analysis. Results are from three separate experiments. Error bars = ± 1 S.E.M.

Chapter 6: Inhibition of *C. albicans* growth by nitrite is alleviated by pretreatment with nitrate or thiocyanate

6.1 Sodium nitrite inhibits *C. albicans* growth in a pH dependent manner

Acidified nitrite is a potent antimicrobial agent where toxicity is a result of the breakdown of nitrite to toxic nitric oxide at acidic pH. The ability of acidified nitrite to inhibit microbial growth has been studied extensively in a number of different species including gut and oral bacteria, and fungi including *C. albicans* (Benjamin et al., 1994; Dykhuizen et al., 1996; Weller et al., 2001; Anyim et al., 2005; Xia et al., 2006). In these studies the antimicrobial effect of nitrite on *C. albicans* was directly correlated with environmental pH. As the pH of growth media decreases, less nitrite is required for effective killing of microbes. Passage through the gut results in exposure to acidified nitrite. It has been proposed that the generation of acidified nitrite, the nitrite generated by symbiotic bacterial reduction of nitrate in saliva, may play a critical role in host defense against ingested pathogens (McKnight et al., 1997).

6.2 Pretreatment with nitrate increases *C. albicans* resistance to sodium nitrite

In order to successfully passage the human stomach to colonize the intestine, *C. albicans* must overcome the toxic effects of acidified nitrite encountered in the stomach. In response to nitrosative stress (acidified nitrite, or nitric oxide), *C. albicans* upregulates a handful of genes, most notably *YHB1* (Hromatka et al., 2005). The transcriptional response of cells treated with either nitrate or thiocyanate is nearly identical to the nitrosative stress response, with *YHB1* being the most highly induced gene under each condition. Because nitrate and thiocyanate are able to generate nearly the same

transcriptional profile as nitric oxide and nitrite, experiments were done to test if pretreating cells with either of these nontoxic compounds could provide protection against the effects of acidified nitrite. In *C. albicans*, deletion of *YHB1* results in hypersensitivity of cells to nitrite (Ullmann et al., 2004). In *S. cerevisiae* overexpression of the *YHB1* regulator Fzf1 results in increased Yhb1 protein levels and increased resistance to the nitric oxide donor DPTA NONOate (Sarver and DeRisi, 2005). Nitric oxide resistance is correlated with Yhb1 protein levels and differences in nitric oxide resistance may simply be a function of the enzymatic activity and overall level of Yhb1 protein in cells.

Pretreatment of *C. albicans* with a sub-lethal dose of hydrogen peroxide (Jamieson et al., 2006) or nitrite (data not shown) or pretreatment of *E. coli* with sub-toxic levels of nitrite (Bower et al., 2009) increases the resistance of cells upon subsequent exposure to that same stress. Thus, exposure to a stress activates a specific adaptive response which results in increased levels of detoxifying and repair proteins. When exposed to that same stress a second time the cells are more resistant to that stress as a result of increased levels of stress response proteins (Berry and Gasch, 2008). In addition to this stress adaptation response, in microbes, three other categories of stress response have been characterized: general stress responses, stochastic switching and predictive responses.

S. cerevisiae generates a general stress response in which ~900 of the total 6,607 predicted genes are significantly up or downregulated in response to a variety of stresses: heat shock, hydrogen peroxide, menadione, diamide, DTT, osmotic shock or nitrogen starvation (Gasch et al., 2000). This overlapping response to stresses may favor cell

survival under conditions where exposure to combinatorial stresses is more likely. This would be more common for a free-living organism living in a constantly changing environment (Gasch et al., 2000). Some microorganisms exist in populations where genetically identical cells can exist in more than one phenotypic state, with one of these states being significantly more stress resistant than the other. This stochastic switching of phenotypes provides greater population fitness in resisting stress by ensuring a sub-population of cells is optimally prepared in the event of an unforeseen change in environmental conditions (Acar et al., 2008). Stochastic switching is beneficial when the cost of maintaining a sub-population of cells optimized for particular environmental changes is lower than the cost of maintaining sensory molecular machinery to continuously monitor the environment (Jansen and Stumpf, 2005; Kussell and Leibler, 2005).

Predictive responses have so far been found in microbes that inhabit environments in which the appearance of certain input signals and stresses are correlated. In the predictive response model, organisms activate specific transcriptional networks in response to stimuli that precede a specific environmental change in a predictable and unidirectional manner. By responding to the preceding stimuli in anticipation of environmental change, an organism is able to increase its fitness within that specific ecological niche (Mitchell et al., 2009).

The mouth is a unique ecological niche for *C. albicans* in that it acts as both a long-term environment niche and a passageway to gastrointestinal colonization. One barrier to GI colonization is surviving passage through the stomach and overcoming the toxic effects of acidified nitrite. This unidirectional route of GI colonization is predicted

to expose *C. albicans* first to nitrate and thiocyanate, which are both actively concentrated in saliva, and then to acidified nitrite in the stomach. Because exposure to nitrate and thiocyanate generate a nearly identical transcriptional response as nitrite and nitric oxide in *C. albicans* pre-exposure of cells to nitrate or thiocyanate should confer a protective effect against subsequent nitrite challenge.

To test whether pretreatment with nitrate protects cells from subsequent nitrite challenge, the wild-type strain SN152 was grown in rich yeast media at pH 6.05 for 4 hours with or without 10 mM nitrate. Cells were then treated with nitrite, incubated for an additional 3 hours and then the concentration of each cell culture was determined by measuring OD₆₀₀. Cells pretreated with nitrate showed greater growth at higher concentrations of nitrite compared to the non-pretreated cultures (Figure 6-1).

Our microarray analysis showed a handful of other genes that were upregulated, or downregulated under all tested conditions. To determine whether these genes contribute to this protective response, I measured growth of *cta4Δ/Δ* and *yhb1Δ/Δ* mutant strains in response to nitrite with or without nitrate pretreatment. The results in Figure 6.1 show the growth for *yhb1Δ/Δ* or *cta4Δ/Δ* strains in media of varying nitrite concentrations is not significantly different between nitrate pretreated and non-pretreated cultures. The data in Figure 6.1, for each condition, was normalized by setting 0 mM nitrite treated cell cultures equal to 1.0. The actual OD₆₀₀ values are listed in Table 2 of Appendix E.

The ability of nitrate to directly protect against nitrite was also tested by adding nitrate just prior to nitrite challenge. Growth of cells treated with nitrate just prior to nitrite challenge grew identically as non-pretreated cells and showed no increased

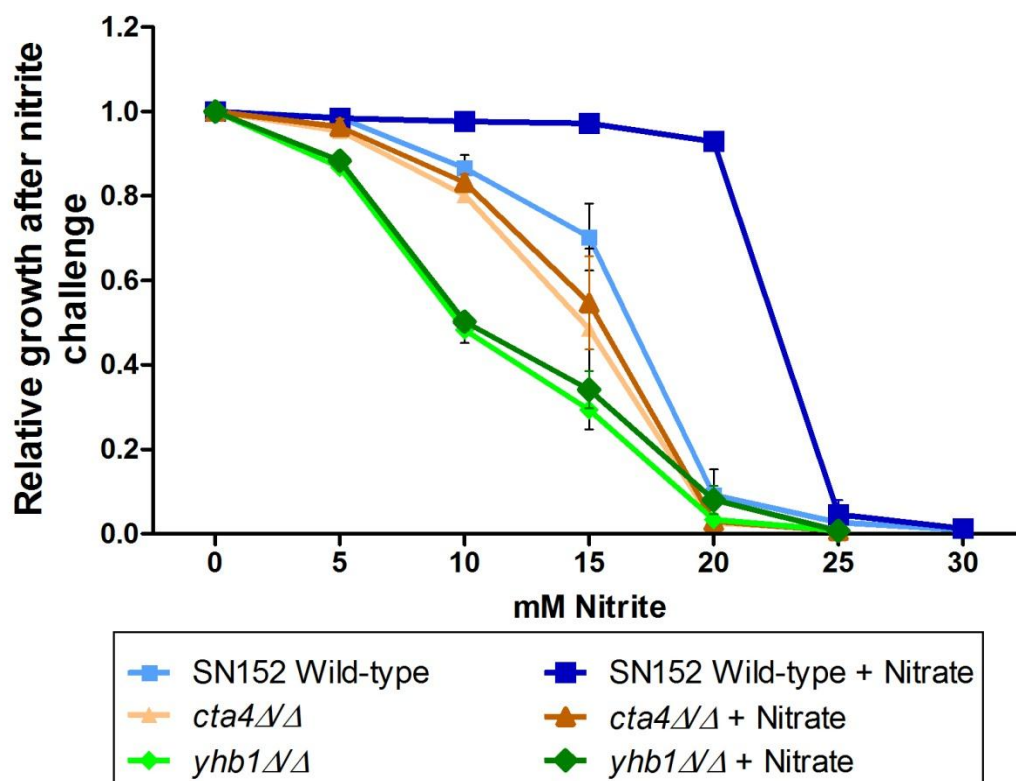


Figure 6-1. Growth inhibition of *C. albicans* strain SN152 (wild-type), *cta4*Δ/Δ and *yhb1*Δ/Δ by nitrite with or without nitrate pretreatment in YEPD, pH 6.05

Tube cultures of *C. albicans* deletion mutants *yhb1* Δ/Δ, *cta4*Δ/Δ and the wild-type parent strain SN152 were grown in rich yeast media (YEPD), adjusted to pH 6.05, with or without 10 mM NaNO₃ for 4 hours. Cultures were then challenged with increasing amounts of nitrite, grown for an additional 3 hours and growth inhibition was determined by measuring OD₆₀₀. Growth rates for each condition are expressed as a percentage of growth after nitrite treatment compared to control (0 mM nitrite) which is normalized to 1.0. Results are from three separate experiments. Error bars = +1 S.E.M.

resistance to acidified nitrite (data not shown). These results are thus consistent with a model in which the protective effect of nitrate pretreatment was a result of Cta4-dependent increase in Yhb1 protein levels and subsequent enhanced detoxification of nitric oxide generated from acidified nitrite rather than a direct protective effect of nitrate itself.

6.3 Pretreatment with nitrate or thiocyanate protects against nitrite challenge under physiological pH and at physiological concentrations

Pretreatment with nitrate increases the resistance of *C. albicans* to nitrite. In the previous experiments I used 10 mM nitrate to pretreat cells, which is only physiologically relevant after consumption of high amounts of nitrate. Also, because these growth assays were done at pH 6.05, nitrite levels well above the normal physiological range were needed to effectively inhibit *C. albicans* growth. To test whether this pretreatment phenomenon occurs under more physiologically relevant conditions, I repeated the growth assays and pretreated cell cultures with either 1 mM nitrate or 1 mM thiocyanate, both concentrations well within the normal physiological range, in media pH adjusted to pH 3.5. The growth of cells was tested at normal salivary and gastric nitrite levels, which have been reported to range between 60-110 μ M or 330 μ M under fasting conditions, up to 760-3300 μ M after nitrate intake (Sádecká and Polonský, 2003; Lundberg and Govoni, 2004; Björne et al., 2006).

Overnight cultures of wild-type cells (SN152) were diluted into rich yeast media pH adjusted to pH 3.5 and 1 mM NaNO₃, 1 mM NaSCN or 1 mM NaCl (control) was added and the cultures were incubated for 4 hours, treated with 0-700 μ M nitrite, grown

for 3.5 additional hours and the concentration of each cell culture was determined by measuring OD₆₀₀. When challenged with acidified nitrite, pretreatment with either nitrate or thiocyanate increased growth of *C. albicans* over the non-pretreated control (Figure 6-2). While both nitrate and thiocyanate were able to increase *C. albicans* resistance to nitrite, thiocyanate was more effective than nitrate in enhancing nitrite resistance. This result is consistent with the result in Figure 5-2 that shows thiocyanate induces greater levels of *YHB1* mRNA transcript at pH 3.5 compared to nitrate. The greater protection from nitrite challenge when pretreated with thiocyanate is most likely the result of greater Yhb1 protein levels.

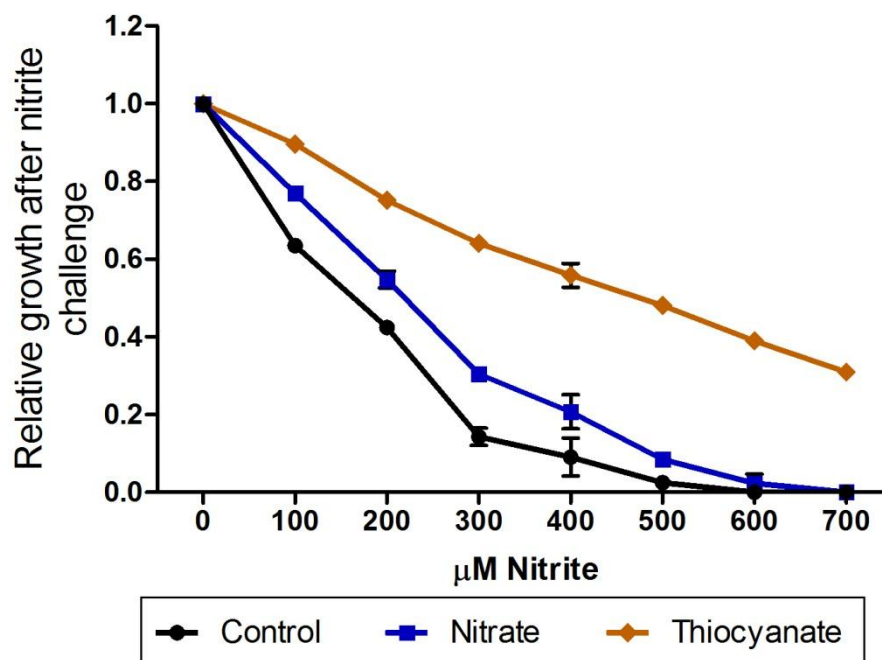


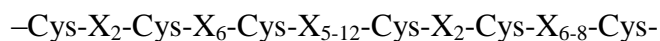
Figure 6-2. Growth inhibition of *C. albicans* strain SN152 by nitrite with nitrate or thiocyanate pretreatment in YEPD, pH 3.5

Tube cultures of *C. albicans* SN152 wild-type strain were grown in rich yeast media (YEPD), adjusted to pH 3.5 with HCl and pretreated with 1 mM nitrate or 1 mM thiocyanate for 4 hours. Cultures were then treated with increasing amounts of nitrite, grown for an additional 3.5 hours and growth inhibition was determined by measuring OD₆₀₀. Growth rates for each condition are expressed as a percentage of growth after nitrite treatment compared to control (0 mM nitrite) which is normalized to 1.0. Results are from three separate experiments. Error bars = ± 1 S.E.M.

Chapter 7: Cta4 forms a homodimer and binds to the NORE in vivo

7.1 Cta4 binds to the NORE region of the *YHB1* promoter in vivo

CTA4 is necessary for *YHB1* induction by nitric oxide, nitrite, nitrate and thiocyanate. Cta4 was initially identified as one of five putative transcription factors that bind to the nitric oxide-responsive element (NORE) in vitro (Chiranand et al., 2008). Of these five transcription factors, only deletion of *CTA4* results in loss of *YHB1* induced expression in response to nitric oxide and nitrite. Based on sequence homology, *CTA4* has been classified as a binuclear zinc binding $[Zn(II)_2Cys_6]$ or zinc cluster protein. This class of proteins is exclusively fungal, typically bind constitutively to target DNA and is necessary for transcriptional activation of target genes (MacPherson et al., 2006). Zinc cluster proteins are defined by a highly conserved:



motif (Campbell et al., 2008). The six cysteine residues bind two zinc atoms which coordinate protein folding and DNA binding.

To determine if Cta4 binds to the *YHB1* promoter in vivo, I performed a chromatin immunoprecipitation assay (ChIP) using a *C. albicans* strain with one copy of *CTA4* deleted and the other copy with a 9xMyc epitope tag fused to the C-terminus of Cta4 (*Cta4Δ/Cta4_{9xMyc}*). This strain with one copy of *CTA4* with a 9xMyc tag appears to be fully functional based on phenotypic analysis. Cells were grown to mid-log phase in rich yeast media and treated with or without 10 mM nitrite at pH 6 for 15 minutes, then treated with formaldehyde to chemically cross-link DNA to proximal proteins. To determine whether Cta4_{9xMyc} binds to the *YHB1* promoter was assessed by using DNA

that co-precipitated with Cta4_{9xMyc} as a template for PCR using primers that flank the NORE to generate a 241 bp PCR product. The results of this experiment (Figure 7-1) show that after exposure to nitrite, Cta4_{9xMyc} is bound to the *YHB1* promoter in vivo. Cta4_{9xMyc} was also bound to the *YHB1* promoter in untreated cells, indicating Cta4 occupies the *YHB1* promoter in the presence or absence of nitrite. No significant PCR product was present when ChIP was performed using non-myc tagged cells (Figure 7-1) or when using primers ~2.5 kb upstream or downstream of the NORE (data not shown).

7.2 Cta4 forms a homodimer in vivo

The *Candida albicans* genome database contains 70 putative or verified zinc cluster transcription factors that regulate a vast array of cellular processes including multi-drug resistance, cell wall composition, sugar and protein metabolism, and response to weak acid stress (Maicas et al., 2005). Zinc cluster proteins typically regulate transcription of target genes by binding to specific DNA sequences as monomers, heterodimers or homodimers. Zinc cluster proteins that bind as homodimers typically bind to DNA sequences that contain symmetrical CGG triplet pairs with 0-23 nucleotide spacing in between the triplet pair (CGG-N₀₋₂₃-GCC) (MacPherson et al., 2006). Other zinc cluster proteins can bind as homodimers or heterodimers, and others like the *S. cerevisiae* Stb5 protein bind to DNA only as heterodimers with other zinc cluster proteins (Akache et al., 2004). The nitric oxide-responsive element (NORE) sequence (TATTACCGTCGG) contains an everted symmetric CGG triplet pair with a single nucleotide spacing between the triplet pair. Based on the NORE sequence and binding of

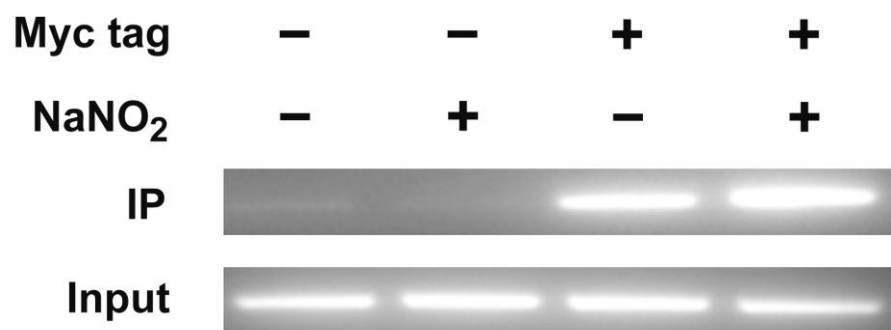


Figure 7-1. Cta4 binds in vivo to the NORE element of the *YHB1* promoter

Chromatin Immunoprecipitation (ChIP) assay using *C. albicans* strain SN152 expressing a single copy Cta4 with a 9xMyc epitope tag at the C-terminus (Cta4_{9xMyc}). Cells were exposed to 0 or 10 mM nitrite for 15 minutes. DNA and Cta4_{9xMyc} complexes were cross-linked with formaldehyde, cells were lysed and DNA was sheared by sonication to ~500 bp fragments - checked by agarose gel electrophoresis. Immunoprecipitation (IP) of Cta4_{9xMyc} was done using Protein G Sepharose beads coated with an anti-myc antibody. Immunoprecipitated DNA was purified by phenol extraction and used as a template for PCR using primers that flank the NORE sequence of the *YHB1* promoter. Input is the PCR product from sample DNA taken before IP. Cells expressing Cta4 without the 9xMyc epitope tag were used as a negative control.

other Zn₂Cyc₆ proteins, Cta4 would be predicted to bind to the NORE as a homodimer to regulate transcription of *YHB1*. To determine whether Cta4 forms a homodimer, I adopted a co-immunoprecipitation strategy to test for physical protein-protein interaction. To do this I took advantage of the diploid chromosome number of *C. albicans* and tagged each chromosomal copy of *CTA4* at the C-terminus with either the 9xMyc or the 6xHA epitope (Cta4_{9xMyc}/Cta4_{6xHA}). Expression of each tagged version of Cta4 was confirmed by Western blot using either anti-HA or anti-Myc antibodies.

This double tagged strain (Cta4_{9xMyc}/Cta4_{6xHA}) was grown in rich yeast media to mid-log phase, cells lysed and Cta4 immunoprecipitated using immobilized Sepharose beads coated with an anti-Myc antibody. Western blot analysis of immunoprecipitated proteins using the anti-HA antibody shows that the Cta4_{6xHA} fusion protein co-immunoprecipitated with the 9xMyc tagged Cta4 (Figure 7-2). Immunoprecipitation of the Cta4_{6xHA} fusion protein occurred only with anti-Myc coated Sepharose beads. No signal was detected using Sepharose beads without anti-Myc antibody (data not shown). Reversing the order of antibodies used for immunoprecipitation and Western blot (i.e., using anti-HA for IP and anti-Myc for Western blot) produced similar results (data not shown). I also tested for heterodimer formation of Cta4 with four other zinc cluster transcription factors (Stb5, War1, Zcf29 and Mrr1) that were identified along with Cta4 by Chiranand et al. (Chiranand et al., 2008), based on the ability of these proteins to bind to the NORE in vitro. For this assay I generated four strains: *CTA4*_{6xHA}/+*STB5*_{9xMyc}, *CTA4*_{6xHA}/+*WAR1*_{9xMyc}, *CTA4*_{6xHA}/+*ZCF29*_{9xMyc}, *CTA4*_{6xHA}/+*MRR1*_{9xMyc}. Co-

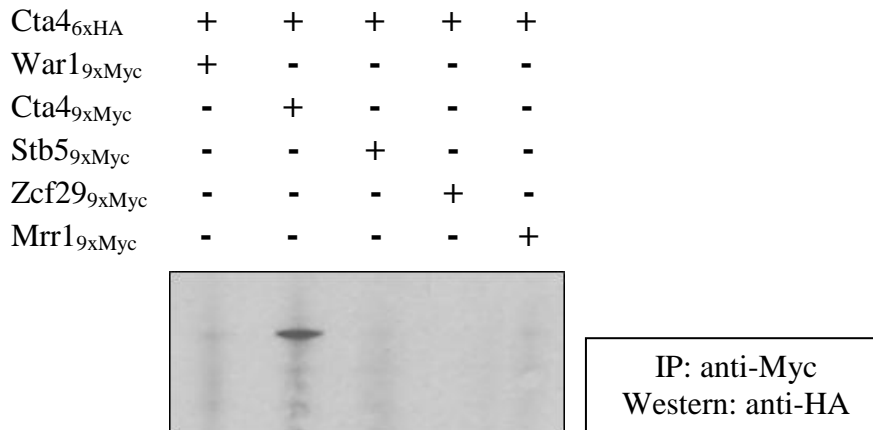


Figure 7-2. Cta4 forms a homodimer in vivo and does not form heterodimers with the other NORE binding proteins.

All five NORE binding proteins were tagged with a 9 copies of Myc at the C-terminus. A single genomic copy of *CTA4* in all of these strains was then tagged with 6 copies of the HA epitope (Cta4_{6xHA}). Strains expressing different combinations of tagged proteins are specified by + or – above the figure. Cells were grown in liquid media, lysed and immunoprecipitated using an anti-Myc antibody bound to Protein G Sepharose beads. Immunoprecipitates were then eluted from the beads, separated by SDS-PAGE and transferred to nitrocellulose membrane. Immunoprecipitated proteins were identified by Western blot using anti-HA antibody.

immunoprecipitation experiments using these strains did not detect any significant interactions with Cta4 (Figure 7-2). These results are consistent with the formation of a homodimer, but this does not exclude the possibility that Cta4 forms a heterodimer with other zinc cluster proteins.

Chapter 8: Analysis of Cta4 as NOx sensor and Cta4-interacting proteins

8.1 Cta4 is a member of the zinc cluster family of transcription factors

Cta4 is a zinc cluster protein that forms a homodimer and constitutively binds to the NORE. Based on the highly conserved zinc cluster motif and similarity in transcriptional regulation of other well characterized zinc cluster transcription factors, Cta4 is predicted to have three primary functional domains: DNA-binding domain, regulatory domain, and activation domain (MacPherson et al., 2006) (Figure 8-1).

The DNA-binding domain is further subdivided into three regions: zinc finger, linker, and dimerization region. The zinc finger contains the –Cys-X₂-Cys-X₆-Cys-X₅₋₁₂-Cys-X₂-Cys-X₆₋₈-Cys- motif that recognizes the CGG triplet pairs. The linker is necessary for correct positioning of the zinc cluster to enable binding to the CGG triplets in the correct orientation and specifies spacing between the triplet pairs (Reece and Ptashne, 1993). The dimerization region forms coiled coil structures either as homodimers or heterodimers (MacPherson et al., 2006). The regulatory domain, also known as the negative regulatory domain or ligand binding domain (Näär and Thakur, 2009), shows very little sequence homology among proteins in this family. The function of this domain varies between zinc cluster proteins. In *S. cerevisiae*, deletion of the regulatory region of Leu3 (Friden et al., 1989) or Pdr1 (Kolaczowska et al., 2002) results in constitutive transcriptional activation. In contrast, *S. cerevisiae* Gal4 protein lacking the regulatory region is still able to regulate transcription similar to the wild-type protein (Ding and Johnston, 1997). Deletion of parts of the *C. albicans* Mrr1 regulatory



Figure 8-1 Functional domains of zinc cluster proteins (not drawn to scale)

Zinc cluster proteins are functionally divided into three domains. The DNA binding domain contains the canonical –Cys-X₂-Cys-X₆-Cys-X₅₋₁₂-Cys-X₂-Cys-X₆₋₈-Cys- motif, linker and dimerization region. The regulatory domain is also referred to as the negative regulatory domain, or ligand binding domain, and is followed by the activation domain.

region, however, results in a non-inducible, non-transcriptionally active protein (Schubert et al., 2011).

The activation domain, also called the acidic domain, typically contains an overall negative charge and typically is necessary for transcriptional activation (Schjerling and Holmberg, 1996; Turcotte et al., 2004) by directly interacting with transcriptional machinery (Melcher and Johnston, 1995).

Many zinc cluster proteins constitutively occupy the promoter regions of target genes and regulate transcription by at least two known mechanisms. Zinc cluster transcription factors can act as both a sensor and transcriptional activator (Sellick and Reece, 2005). Small molecules that bind to the regulatory region cause a conformational change that expose the activation domain and results in transcriptional activation. Transcriptional regulation can also occur through the action of a negative regulatory protein, which binds to the activation domain of the zinc cluster transcription factor and blocks access of transcriptional machinery to the activation domain. In the presence of a specific activating ligand, the protein complex disassociates and exposes the activation domain to transcriptional machinery, resulting in transcriptional activation (Sellick and Reece, 2005).

8.2 Cta4 constitutively activates transcription of target genes when heterologously expressed in *S. cerevisiae*.

The most extensively studied zinc cluster transcription factor is Gal4 from *S. cerevisiae*. Gal4 regulates transcription of genes involved in galactose metabolism that contain a specific Upstream Activation Sequence (UAS). In the absence of galactose,

Gal4 is inactive as a result of the interaction with the negative regulator Gal80p, which binds to the activation domain in the absence of galactose and prevents transcriptional activation (Ding and Johnston, 1997). In the presence of galactose, inhibition of Gal4 by Gal80 is relieved, which results in transcriptional activation. Gal4, when expressed in a *gal80Δ/Δ* mutant strain (Torchia et al., 1984) or heterologously in mammalian cells (Kakidani and Ptashne, 1988) which lack the Gal80 protein, results in constitutive transcriptional activation of a reporter gene driven by the UAS in the absence of galactose. The zinc cluster transcription factor Leu3 in *S. cerevisiae*, which regulates genes involved in leucine biosynthesis, is positively regulated by α -isopropylmalate (α -IPM) and is bound to target gene promoters under all conditions (Kirkpatrick and Schimmel, 1995). However, when expressed in mammalian cells, Leu3 is unable to activate transcription of a reporter gene unless α -IPM is externally added to the medium (Guo and Kohlhaw, 1996). When leucine levels are high, the activation domain of Leu3 is masked by intramolecular binding to its regulatory domain. In the presence of α -IPM, the activation domain is unmasked, allowing interaction with transcriptional machinery and transcriptional activation of target genes (Wang et al., 1999). This type of direct sensing of an inducing ligand is also found in other zinc cluster transcription factors. Put3 can directly bind proline (Des Etages et al., 2001), Hap1 can directly bind heme (Hon et al., 2003) and Ppr1 binds orotic acid (Flynn and Reece, 1999). All of these ligand binding events result in positive regulation of these proteins and transcriptional activation.

In *C. albicans* *CTA4* is necessary for *YHB1* induction by nitric oxide, nitrite, nitrate and thiocyanate. To determine if *CTA4* acts both as a sensor and transcriptional

activator, and is therefore sufficient for *YHB1* induction by nitric oxide or nitrate, I expressed *CTA4* and examined its function in a *S. cerevisiae* heterologous protein expression system. A plasmid with a reporter gene (*lacZ*), driven by the *C. albicans* *YHB1* promoter ($P_{CaYHB1-lacZ}$), was transformed into *S. cerevisiae*. The β -galactosidase activity of these cells treated with nitrite, nitrate or untreated (control) was measured to determine basal promoter activity in the absence of *CTA4*. The activity of the promoter under both control and inducing conditions was low, indicating that the cis-acting element of the *C. albicans* *YHB1* promoter (NORE) is specific to *C. albicans* and not responsive to nitric oxide or nitrate in *S. cerevisiae*. This was expected because Fzf1, the known transcriptional regulator of the *S. cerevisiae* *YHB1*, is a different class of zinc binding protein (C_2H_2) that binds to a consensus motif CTATCAYTT (DeRisi et al., 2000) different from the NORE. Another plasmid was constructed that expresses a codon-optimized 9xMyc tagged version of *CTA4* (*CTA4*_{9xMyc}). This was done by replacing all CTG codons, which code for serine in *C. albicans* but leucine in *S. cerevisiae*, to alternate serine codon triplets and inserting DNA encoding the 9xMyc tag before the stop codon of *CTA4*. A similarly tagged version of Cta4 is fully functional in *C. albicans*. I expressed *CTA4*_{9xMyc} from a *tetO-CYC1* promoter under control of a hybrid *tetR*-VP16 (tTA) activator protein – a version of the Tet-off system. tTA binds to *tetO-CYC1* and activates transcription in the absence of tetracycline. The addition of tetracycline to the medium results in a loss of binding to *tetO-CYC1* and loss of transcriptional activation. This allows *CTA4* expression levels to be controlled by the adding or withholding of tetracycline from the growth medium (+Tet: low Cta4 levels, -

Tet: high Cta4 levels) (Garí et al., 1997). Protein levels of Cta4_{9xMyc} were monitored by Western blot using an anti-Myc antibody.

To test whether *CTA4* is sufficient for nitrite or nitrate mediated induction of the *P_{YHBI}-lacZ* reporter gene, the β -galactosidase activity of nitrite, nitrate and untreated (control) cells was measured in the presence or absence of tetracycline. As expected, β -galactosidase activity of the untreated samples was reduced when tetracycline was added (Figure 8-2). Under each of the conditions tested, the β -galactosidase activity in the nitrite- or nitrate-treated samples was nearly identical to the control. Addition of 1 μ g/ml tetracycline resulted in no detectable Cta4_{9xMyc} expression by Western blot and no difference in *lacZ* expression levels compared to strains carrying only the *P_{CaYHBI}-lacZ* reporter gene without *CTA4*_{9xMyc} (data not shown). A range of tetracycline concentrations was tested (0, 0.1, 0.33, 0.5, 0.7 and 1.0 μ g/ml). At each of the tetracycline concentrations tested, β -galactosidase activity in the nitrite- and nitrate- treated samples was nearly identical to the control (data not shown).

These results suggest that Cta4 transcriptional activation of target genes is controlled by additional factors that are specific to *C. albicans* and absent in *S. cerevisiae*. Unlike the auto-inhibition seen with Leu3, this type of transcriptional regulation of a zinc cluster protein appears to be more similar to Gal4 regulation in which the zinc cluster transcription factor is negatively regulated by another factor. Based on this data we propose a model of transcriptional regulation in which an unknown factor ('X') binds to and negatively regulates Cta4 activity, and only in the presence of the inducer, nitric oxide, nitrate or thiocyanate, is this repression removed allowing transcriptional activation (Figure 8-3).

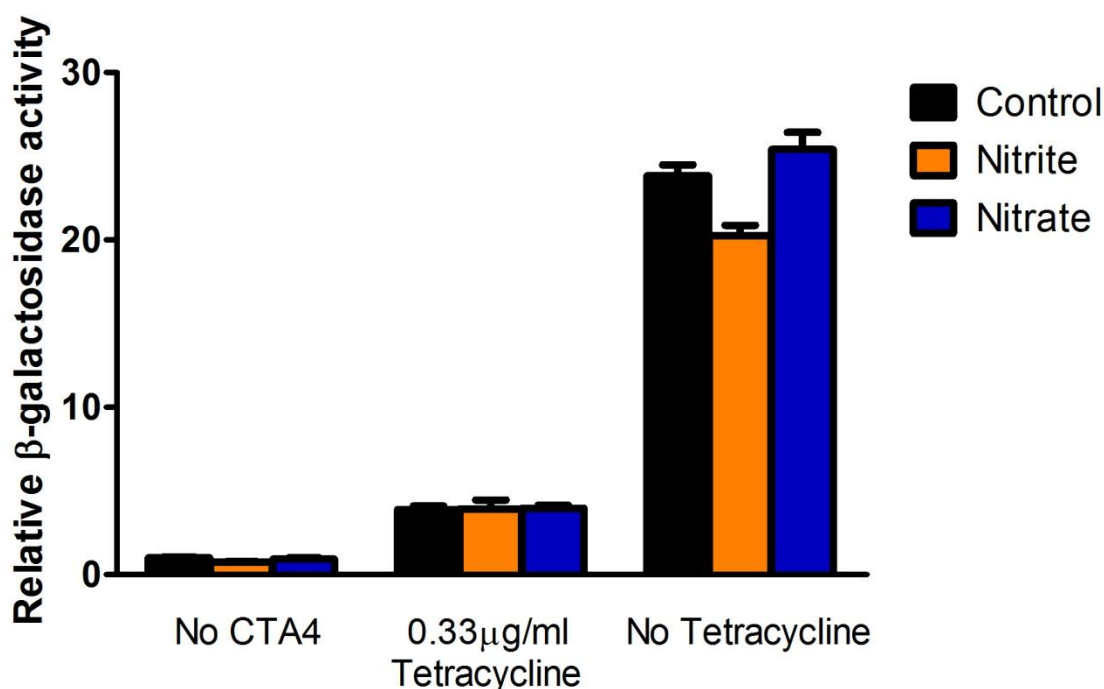


Figure 8-2. Heterologous expression of *CTA4* in *S. cerevisiae*

A centromeric plasmid containing a 500 bp fragment of the *C. albicans* *YHB1* promoter (containing the NORE) driving *lacZ* expression (P_{YHB1} -*lacZ*) was transformed into *S. cerevisiae* strain BY4742. A codon optimized version of *CTA4*, expressed from a tetracycline regulatable promoter (-tet high Cta4, + low Cta4) was transformed into strain carrying P_{YHB1} -*lacZ*. Both strains were grown with or without tetracycline in appropriate drop out media and treated with nitrite, nitrate or untreated (control). Data were normalized between each experiment by expressing relative β -galactosidase activity as the fold increase in expression above control (No CTA4). Results are from three separate experiments. Error bars = ± 1 S.E.M.

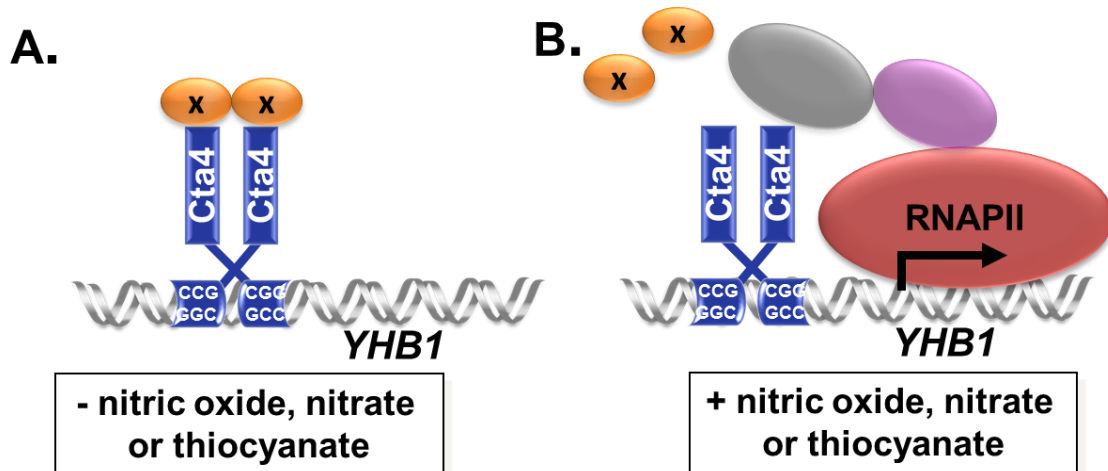


Figure 8-3. Proposed model of Cta4 transcriptional regulation

A. In the absence of inducing compounds (- nitric oxide, nitrate or thiocyanate) Cta4 binds as a dimer to the *YHB1* promoter with each Cta4 protein binding to one of the CCG triplets of the NORE sequence (CCGTCGG). Transcriptional activation is prevented by the binding of an unknown factor ('x'). B. In the presence of any of the activating compounds (+ nitric oxide, nitrate or thiocyanate) Cta4 remains bound to the NORE and transcriptional repression by 'x' is lifted, by an unknown mechanism, allowing access of the transcriptional machinery (RNAPII) to Cta4 resulting in transcription of *YHB1*.

8.3 Cta4-TAP tag with a 3xFLAG epitope at the N-terminus and a ProtA epitope at the C-terminus is functionally expressed in *C. albicans*

Cta4 binds to the *YHB1* promoter in vivo and in vitro most likely as a homodimer. Heterologous expression of *CTA4*_{9xMyc} in *S. cerevisiae* resulted in constitutive activation of a reporter gene. Based on these data and our knowledge of zinc cluster transcription factor regulation, I propose a model in which a negative regulator binds to Cta4 and inhibits transcriptional activation. Only when the inducing ligands are present is this inhibition relieved, allowing transcription of *YHB1*. To identify any proteins that bind to Cta4, I adapted a tandem affinity purification (TAP) method to purify Cta4 and any associated proteins. The TAP method involves expressing a dual epitope tagged protein, at the N- or C-terminus, and purifying the protein complex in two successive purification steps (Puig et al., 2001). Proteins that are co-purified with the tagged protein can then be identified by mass spectrometry.

To perform TAP, I first constructed various affinity tags fused to either the N- or C-terminus of Cta4 and tested for functionality by transforming these fusion proteins into a *cta4Δ/Δ* strain expressing the *YHB1-lacZ* reporter gene. Because Cta4 contains a DNA binding domain at the N-terminus and an activation domain at the C-terminus, different combinations of tags were constructed and tested to find ones that were able to both maintain Cta4 function and be effectively purified away from lysate at a concentration sufficient to be visualized by SDS-PAGE. After testing various combinations, the best combination found consisted of the 3xFLAG tag (MDYKDHDGDYKDHDIDYKDDDK) at the N-terminus and the ProtA tag at the C-terminus. The ProtA tag is a 125 amino acid segment with two consecutive segments of

the *Staphylococcus aureus* protein A that binds with high affinity to the Fc region of most IgG antibodies (Rigaut et al., 1999). A TEV (tobacco etch virus) protease cleavage site (ENLYFQG) was inserted between Cta4 C-terminus and the ProtA tag in order to allow non-denaturing release of Cta4 from IgG coated beads by proteolytic cleavage. A 9 amino acid flexible spacer was also added between the Cta4 C-terminus and the TEV cleavage site. This spacer was found to be necessary to maintain proper Cta4 function, possibly by creating greater distance between the ProtA tag and the C-terminus allowing transcriptional machinery to access the activation domain at the Cta4 C-terminus (Figure 8-4).

8.4 Purification of Cta4 by tandem affinity purification did not identify any additional associated proteins by mass spectrometry analysis

To purify TAP tagged Cta4 along with any additional interacting proteins, cells expressing tagged Cta4 were grown in rich yeast media under non-inducing conditions to $OD_{600} \approx 1.5$. Cells were lysed and tagged proteins were purified by first incubating the lysate with IgG Sepharose 6 Fast Flow resin. Unbound material was washed off and tagged proteins were released from the beads by incubating with TEV protease. The proteins released from the IgG coated beads were then incubated with anti-FLAG antibody coated beads, unbound material was washed off and bound proteins were released from the beads by adding excess FLAG peptide. In parallel, I prepared cell extracts and performed the identical purification procedure using a strain without native or tagged *CTA4* (*cta4Δ/Δ*). The eluted proteins from *cta4Δ/Δ* and Cta4-TAP strains were separated, side-by-side, by SDS-PAGE and visualized by silver stain (Figure 8-5).

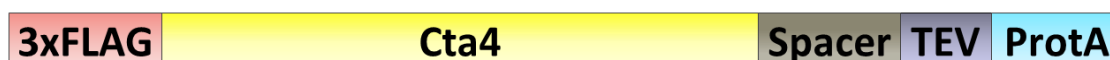


Figure 8-4. Schematic of Cta4-TAP used for tandem affinity purification (TAP) of Cta4 and associated proteins

Diagram showing Cta4-TAP which consists of a 3xFLAG tag at the N-terminus and the ProtA tag at the C-terminus. The tobacco etch virus (TEV) protease cleavage site (ENLYFQG) is incorporated between the ProtA tag and Cta4 protein to allow proteolytic release of Cta4 away from ProtA immobilized by IgG Sepharose beads during the first purification step. The addition of a 9 amino acid flexible spacer was necessary to maintain proper Cta4 function.

This assay was repeated three times and in each prep there were four bands that were consistently present in the Cta4 tagged lane, but not in the *cta4Δ/Δ* lane, including the band corresponding to Cta4 based on the predicted molecular weight (red arrow Figure 8-5). Some bands, such as the band just below Cta4 in Figure 8-5, were present predominantly in the Cta4 tagged lane and nearly absent in the *cta4Δ/Δ* lane in some preps – such as the prep shown in Figure 8-5. While in other preps these bands were equally represented in both lanes. These four protein bands were excised from the gel and submitted to the Stanford PAN Facility for identification by mass spectrometry. Peptide sequences obtained from MS+MS/MS analyses were identified by BLAST search using the *Candida* Genome Database (www.candidagenome.org). The band corresponding to the TAP tagged Cta4 protein based on molecular weight prediction was positively identified as Cta4. The bands labeled #1 and #2 were also both identified as Cta4. It is unclear why a sub fraction of Cta4 would migrate more slowly during SDS-PAGE. It may be that Cta4 is being covalently modified resulting in slower migration on the gel. This, however, seems unlikely because the only peptide fragments identified by MS correspond to the Cta4 protein sequence with no additional mass. A more likely explanation is that the higher molecular weight band is a result of Cta4 dimer formation.

The mass spectrometry data from the protein band labeled #3 in Figure 8-5 identified two proteins, Gcf1 (M.W. 28.5 kDa) and Rps6a (M.W. 27.1 kDa). Gcf1 is an HMG box-containing mitochondrial protein. It binds to both mitochondrial and genomic DNA, it is predicted to have a role in mitochondrial genome maintenance, and is annotated in the *Candida* Genome Database as being an essential gene. Rps6a is a

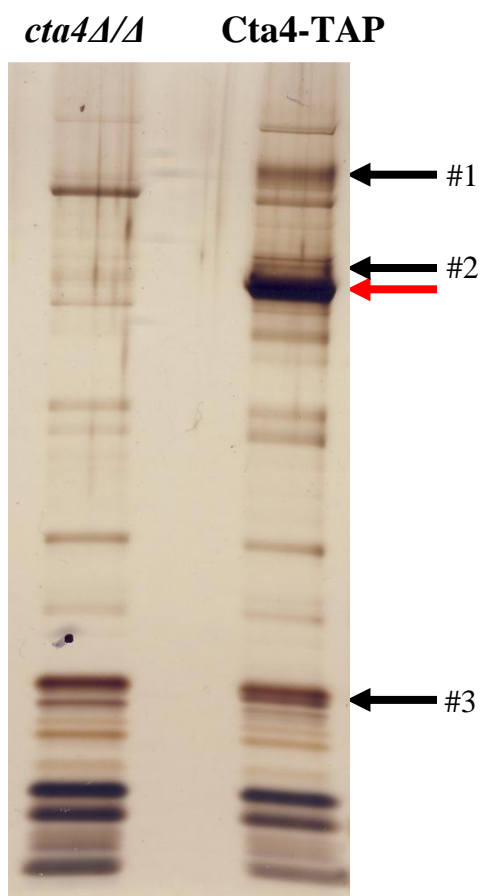


Figure 8-5 Silver stain analysis of tandem affinity purified proteins from a Cta4-TAP and *cta4Δ/Δ* strain

Lysate from Cta4-TAP tagged and a *cta4Δ/Δ* strain were subjected to sequential purification steps. Eluates from the final purification step were separated by SDS-PAGE and visualized using PageSilver™ Silver Staining Kit (Fermentas). Numbered black arrows indicate protein bands present in Cta4-TAP eluate after the final purification step and absent in the *cta4Δ/Δ* final eluate. Red arrow indicates the predicted molecular weight of TAP tagged Cta4. All protein bands indicated with arrows were further analyzed by MS + MS/MS.

protein component of the small ribosomal subunit. Both Gcf1 and Rps6a have mammalian homologs (not fungal specific). Because neither of these proteins appeared to be good candidates as a negative regulator of Cta4, I did not further pursue further investigation of either of these proteins.

Chapter 9: Conclusions and Future Direction

This work identified and characterized a novel transcriptional response in *Candida albicans* to nitrate and the pseudohalide thiocyanate. Treating cells with nitrate or thiocyanate produces a nearly identical transcriptional response compared to cells treated with nitrite or nitric oxide. Because nitrate and thiocyanate are not toxic and not utilized by *C. albicans* for any known metabolic purpose, we looked at this phenomenon in the context of *C. albicans*/host interaction as a possible adaptive response. We have thus identified a unique transcriptional response in *C. albicans* that promotes nitrosative stress resistance. This response is predicted to facilitate *C. albicans* survival during transit through the stomach in order to successfully colonize the gastrointestinal tract of a human host (Figure 9-1).

In this work I show that pretreatment of *C. albicans* with physiologically relevant concentrations of nitrate or thiocyanate promotes nitrite stress tolerance and that this response is absent in the budding yeast *S. cerevisiae*. This response is an example of an anticipatory stress response in which a preceding signal predicts the appearance of a subsequent stress. In the context of gastrointestinal colonization, the appearance of nitrate and thiocyanate, both actively concentrated in the saliva, will always precede the formation of nitric oxide in the stomach from the acidification of nitrite. In this system, because the sequence of appearance of the signal always precedes the stress, organisms that couple the responses to both the signal and subsequent stress gain a fitness advantage. The predictable sequence of events during colonization acts as a directed

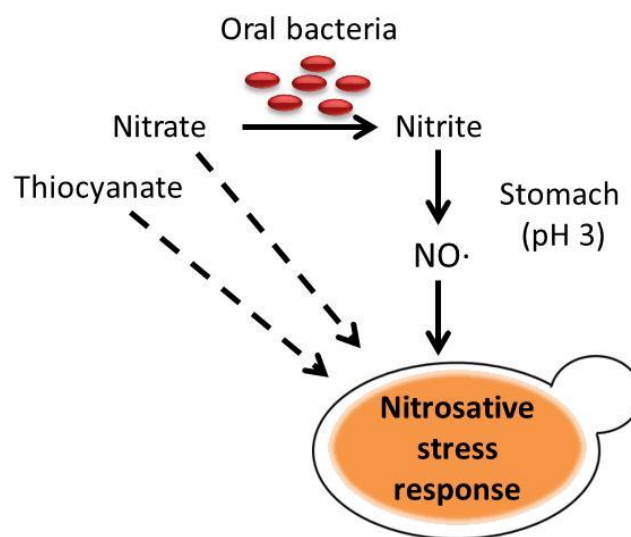


Figure 9-1. Induction of nitrosative stress response genes in *C. albicans* in response to nitrate and thiocyanate confers protection against future nitrosative stress generated by the acidification of nitrite in the stomach

C. albicans is exposed to nitric oxide stress when nitrite, generated by oral bacteria from nitrate, is acidified in the stomach (pH 3). This exposure to nitric oxide induces nitrosative stress response genes to counteract the toxic effects of nitric oxide. Prior to entry into the stomach, *C. albicans* will be exposed to constituents of saliva which includes nitrate and thiocyanate, both actively concentrated by salivary glands. In this environment the appearance of nitrate and thiocyanate always precedes the appearance of NO·. By coupling the sensing of nitrate and thiocyanate (dashed arrows) with nitrosative stress response genes, *C. albicans* is able to predict the appearance of imminent nitrosative stress which increases its resistance to nitric oxide.

evolution experiment in which those within the population that are able form a link between the signal and stress gain a growth advantage.

Based on our in vitro data we predict that pre-exposure of *C. albicans* to nitrate or thiocyanate will increase colonization in the gut and intestine. Testing this in an in vivo mouse model of colonization would be most ideal to support our hypothesis. However, because mice are not natural carriers of *C. albicans*, the method of establishing *C. albicans* gastrointestinal colonization in mice requires both the pretreatment with antibiotics to deplete the animal of commensal flora, and introduction of *C. albicans* into the animal by oral gavage using a feeding needle or via food and water (Maccallum, 2012). Removal of commensal bacteria by antibiotic treatment prior to *C. albicans* introduction would greatly reduce or eliminate nitrate reduction to nitrite, thus eliminating nitric oxide formation in the gut. Loss of normal nitric oxide formation removes the colonization barrier we are testing for, thereby making traditional methods of *C. albicans* colonization in mice poorly suited to compare colonization rates of strains pretreated and non-pretreated with nitrate or thiocyanate.

An interesting question from this work is the mechanism by which *C. albicans* senses both nitrate and thiocyanate. Unlike nitric oxide that is able to diffuse through the lipid bilayer, nitrate and thiocyanate sensing requires either transport into the cytosol, or extracellular sensing followed by intracellular signal transduction. To identify what genes may be involved in this signal response requires both an assay to identify a mutant phenotype and a mutant strain collection. Unlike *S. cerevisiae*, *C. albicans* is an obligate diploid organism and lacks an exploitable sexual cycle. This hinders standard forward genetic screening approaches. In *C. albicans* forward genetic screens have been

successful in identifying genes that regulate morphogenesis (Uhl et al., 2003; Bharucha et al., 2011), however these screens were only able to identify haploinsufficient genes. Because neither *CTA4* nor *YHBI* is haploinsufficient we reason that regulators of this pathway are also not haploinsufficient.

Because of this, the more common screening approach in the *C. albicans* field is through a reverse genetics approach in which genes of interest are deleted and their phenotypes characterized. A number of labs have generated large scale gene deletion collections in *C. albicans* and made these strains available through the Fungal Genetics Stock Center (www.fgsc.net). These collections have been used to identify novel virulence factors (Noble et al., 2010) and regulators of biofilm formation (Nobile et al., 2012) and to ascribe biological functions to previously unknown putative transcription factors (Homann et al., 2009).

To identify genes involved in nitrate or thiocyanate sensing, strains from the deletion collections can be subjected to nitrite sensitivity growth assays with or without nitrate or thiocyanate pretreatment. Pretreatment with nitrate or thiocyanate confers a growth advantage when challenged with nitrite, so that any strains that show no growth difference in pretreated and non-pretreated growth after nitrite challenge would be good candidates for further investigation. There are two advantages of this assay. The first is that a protocol has already been established that generates highly reproducible results, so that conditions to screen these collections do not need to be developed. The second advantage of this method is differences in nitrite resistance are determined by comparing growth of the same strain challenged with nitrite with or without nitrate or thiocyanate pretreatment. Therefore, any growth difference between deletion strains do not affect

data interpretation of slower or faster growing strains. It is not uncommon for *C. albicans* deletion strains, of the same genetic background, to have different growth rates, whether because of genomic alteration during the transformation process, or because the gene supports normal growth. The obvious caveat of this approach is at this time the deletion collections available now do not include all non-essential genes, so there is a possibility that the gene deletion strains of interest are not part of any current collection. Or it may be that these genes are essential and need to be identified using a different approach.

Induction of *YHB1* by nitric oxide, nitrate and thiocyanate all require the zinc cluster transcription factor Cta4. Heterologous expression of *CTA4* in *S. cerevisiae* results in constitutive activation of a reporter gene driven by the *C. albicans YHB1* promoter, and the addition of the inducing ligands, nitrite or nitrate, did not lead to increased transcriptional activation. I also found that ectopic overexpression of *CTA4* in *C. albicans* from the actin promoter (*ACT1*) results in the constitutive activation of *YHB1-lacZ* expression and the addition of nitrite or nitrate did not increase *YHB1* expression. We hypothesize that the overexpression of Cta4 leads to the titrating out of a negative regulator, which results in constitutive transcriptional activation. Based on these results we propose a model of Cta4 regulation in which transcriptional activation is inhibited by a negative regulator that binds to Cta4 which blocks access of transcriptional machinery to the C-terminal activation domain of Cta4 (Figure 8-3).

I was unable to co-purify the putative negative regulatory by tandem affinity purification. This may be because of technical issues with the affinity purification protocol and proper modifications to the procedure may lead to positive identification of

a Cta4 negative regulator. Alternatively, the binding of a regulator with Cta4 may be transient and involve modification of Cta4 leading to transcriptional activation like the Yap1 protein in *S. cerevisiae*. Yap1 is a basic leucine zipper (bZIP) transcription factor that regulates anti-oxidant gene expression. Yap1 is activated by a transient interaction with the protein Gpx3, which, in response to hydrogen peroxide, results in intramolecular disulfide bond formation, relocation from the cytosol to the nucleus and transcriptional activation of target genes (Delaunay et al., 2002). To identify transiently binding proteins the addition of an in vivo cross-linker may help to facilitate transient binding of complexes.

Nitric oxide sensing has been described in both prokaryotic and eukaryotic systems, and both differ significantly. In prokaryotes, known nitric oxide sensing proteins act as both nitric oxide sensors and transcriptional regulators of nitric oxide responsive genes. Nitric oxide sensors bind either a single iron atom, or an iron-sulfur cluster, and binding of nitric oxide to these prosthetic groups results in activation of target genes. In eukaryotes, however, nitric oxide sensing occurs by at least two distinct mechanisms. The first is regulation of vasodilation in which nitric oxide binds to the heme prosthetic group of soluble guanylate cyclase (sGC) in low nanomolar concentrations. This binding activates guanylate cyclase which converts GTP to the second messenger cGMP (Boon and Marletta, 2005). Nitric oxide sensing has also been shown to occur via S-nitrosylation of specific cysteine residues by covalent addition of nitric oxide ($S-H \rightarrow S-NO$) (Hess et al., 2005). Modification of specific cysteine residues has been shown to regulate proteins with a broad range of functions including caspase

cysteine proteases (Stamler et al., 1992), membrane receptors (Lipton et al., 2002), ion channels (Eu et al., 2000) and transcription factors (Sumbayev et al., 2003).

After Cta4 was identified as regulating nitric oxide induced expression of *YHB1*, prior to discovering the nitrate and thiocyanate response, I tested Cta4 as a candidate for cysteine modification by S-nitrosylation using the biotin switch technique (BST). This technique selectively labels S-nitrosylated cysteine residues with a modified biotin compound that allows for high affinity purification using immobilized streptavidin (Jaffrey et al., 2001). Based on data I obtained using this assay, Cta4 is not S-nitrosylated. Given the data obtained since I performed this assay, it is not surprising that direct nitrosylation does not activate Cta4 activity. It would seem unlikely that Cta4 directly senses nitric oxide, nitrate and thiocyanate, or that Cta4 selectively senses nitric oxide, but activation by nitrate and thiocyanate occurs via a different mechanism.

In *S. cerevisiae* (Sarver and DeRisi, 2005) and *A. nidulans* (Schinko et al., 2010) protein regulators of flavohemoglobin expression have been identified. Interestingly, however, no nitric oxide sensor has been identified in fungi. To identify the nitric oxide sensor in *C. albicans* strains from the deletion collections can be subjected to nitrite sensitivity growth assays with or without nitrite pretreatment. Strains that show no growth difference in pretreated and non-pretreated growth after nitrite challenge would be good candidates for further investigation.

Closing Remarks

This work identified and characterized a nearly identical transcriptional response to nitric oxide, nitrate and thiocyanate in *C. albicans*. Based on this and this and other

data I put forward a hypothesis that nitrate and thiocyanate in saliva act as environmental cues that signal eminent nitrosative stress generated by the acidification of nitrite in the stomach. This type of anticipation response based on preceding environmental cues has only been describes in only a handful of other microbes so far and may be more widespread among microbes. Investigation of other microbes, in the context of their natural environment, may provide more examples of this anticipatory response giving greater understanding of how this type of adaptation evolved and how that adaptation changes the growth dynamics within a microbial population.

C. albicans is a commensal that colonizes numerous niches on a human host and can also be an opportunistic pathogen in individuals with compromised immune function. Understanding how *C. albicans* is able to adapt and respond to various stresses on and in the human body can provide insight into its mode of pathogenicity and its ability to evade immune detection and killing. It also provides an opportunity to study the unique biological aspects of *C. albicans* and how it has evolved from non-commensal ancestors to survive within a human host.

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Appendix A: Strains used in this study

Strain	Genotype	Source
SC5314	Wild-type clinical isolate	(Gillum et al., 1984)
SN152	<i>arg4Δ::dpl200/arg4Δ::dpl200</i> <i>leu2Δ::dpl200/leu2Δ::dpl200</i> <i>his1Δ::dpl200/his1Δ::dpl200</i> <i>URA3/ura3Δ::imm⁴³⁴</i> <i>IRO1/iro1Δ::imm⁴³⁴</i>	(Noble and Johnson, 2005)
16xNORE SN152	<i>hwp1::pAU96-16xNORE-YHB1-lacZ(Nou^R)/HWP1</i> <i>arg4Δ::dpl200/arg4Δ::dpl200</i> <i>leu2Δ::dpl200/leu2Δ::dpl200</i> <i>his1Δ::dpl200/his1Δ::dpl200</i> <i>URA3/ura3Δ::imm⁴³⁴</i> <i>IRO1/iro1Δ::imm⁴³⁴</i>	This study
<i>yhb1Δ/yhb1Δ</i>	<i>yhb1Δ::C.m.LEU2/yhb1Δ::C.d.ARG4</i> <i>arg4Δ::dpl200/arg4Δ::dpl200</i> <i>leu2Δ::dpl200/leu2Δ::dpl200</i> <i>his1Δ::dpl200/his1Δ::dpl200</i> <i>URA3/ura3Δ::imm⁴³⁴</i> <i>IRO1/iro1Δ::imm⁴³⁴</i>	(Chiranand et al., 2008)
<i>cta4Δ/cta4Δ</i>	<i>cta4Δ::C.m.LEU2/cta4Δ::C.d.ARG4</i> <i>arg4Δ::dpl200/arg4Δ::dpl200</i> <i>leu2Δ::dpl200/leu2Δ::dpl200</i> <i>his1Δ::dpl200/his1Δ::dpl200</i> <i>URA3/ura3Δ::imm⁴³⁴</i> <i>IRO1/iro1Δ::imm⁴³⁴</i>	(Chiranand et al., 2008)
<i>Cta4Δ/Cta4^{9xMyc}</i>	<i>cta4Δ::CmLEU2/CTA4-9xMyc-</i>	(Chiranand et al., 2008)

	<p>(<i>ACT1</i>-3'UTR) -<i>CdHIS1</i></p> <p><i>arg4Δ::dpl200/arg4Δ::dpl200</i></p> <p><i>leu2Δ::dpl200/leu2Δ::dpl200</i></p> <p><i>his1Δ::dpl200/his1Δ::dpl200</i></p> <p><i>URA3/ura3Δ::imm</i>⁴³⁴</p> <p><i>IRO1/iro1Δ::imm</i>⁴³⁴</p>	
<i>Cta4</i> _{9xMyc} / <i>Cta4</i> _{6xHA}	<p><i>CTA4</i>-6xHA (<i>ACT1</i>-3'UTR)::<i>C.d.ARG4</i>/<i>CTA4</i>-9xMyc- (<i>ACT1</i>-3'UTR)::<i>C.d.HIS1</i></p> <p><i>arg4Δ::dpl200/arg4Δ::dpl200</i></p> <p><i>leu2Δ::dpl200/leu2Δ::dpl200</i></p> <p><i>his1Δ::dpl200/his1Δ::dpl200</i></p> <p><i>URA3/ura3Δ::imm</i>⁴³⁴</p> <p><i>IRO1/iro1Δ::imm</i>⁴³⁴</p>	This study
<i>cta4Δ/cta4Δ/Cta4</i> -TAP	<p><i>rps1::Cip20-CTA4-TAP(HIS1)/RPS1</i></p> <p><i>cta4Δ::C.m.LEU2/cta4Δ::C.d.ARG4</i></p> <p><i>arg4Δ::dpl200/arg4Δ::dpl200</i></p> <p><i>leu2Δ::dpl200/leu2Δ::dpl200</i></p> <p><i>his1Δ::dpl200/his1Δ::dpl200</i></p> <p><i>URA3/ura3Δ::imm</i>⁴³⁴</p> <p><i>IRO1/iro1Δ::imm</i>⁴³⁴</p>	This study
<i>19.3120Δ/19.3120Δ</i>	<p><i>19.3120Δ::C.m.LEU2/19.3120Δ::C.d.ARG4</i></p> <p><i>arg4Δ::dpl200/arg4Δ::dpl200</i></p> <p><i>leu2Δ::dpl200/leu2Δ::dpl200</i></p> <p><i>his1Δ::dpl200/his1Δ::dpl200</i></p> <p><i>URA3/ura3Δ::imm</i>⁴³⁴</p> <p><i>IRO1/iro1Δ::imm</i>⁴³⁴</p>	This study
<i>ctr2Δ/ctr2Δ</i>	<i>ctr2Δ::C.m.LEU2/ctr2Δ::C.d.HIS1</i>	(Noble et al., 2010)

	<i>arg4Δ::dpl200/arg4Δ::dpl200</i> <i>leu2Δ::dpl200/leu2Δ::dpl200</i> <i>his1Δ::dpl200/his1Δ::dpl200</i> <i>URA3/ura3Δ::imm⁴³⁴</i> <i>IRO1/iro1Δ::imm⁴³⁴</i>	
<i>rhr2 Δ/rhr2Δ</i>	<i>rhr2 Δ::HIS1/rhr2 Δ::ARG4 ura3</i> <i>Δ::λimm⁴³⁴/URA3 arg4</i> <i>Δ::hisG/arg4Δ::hisG</i> <i>his1Δ::hisG/his1Δ::hisG</i>	(Bonhomme et al., 2011)
<i>ssu1Δ/ssu1Δ</i>	<i>ssu1Δ::C.m.LEU2/ssu1Δ::C.d.HIS1</i> <i>arg4Δ::dpl200/arg4Δ::dpl200</i> <i>leu2Δ::dpl200/leu2Δ::dpl200</i> <i>his1Δ::dpl200/his1Δ::dpl200</i> <i>URA3/ura3Δ::imm⁴³⁴</i> <i>IRO1/iro1Δ::imm⁴³⁴</i>	(Noble et al., 2010)
<i>19.5785Δ/19.5785Δ</i>	<i>19.5785Δ::C.m.LEU2/19.5785Δ::C.d.</i> <i>ARG4</i> <i>arg4Δ::dpl200/arg4Δ::dpl200</i> <i>leu2Δ::dpl200/leu2Δ::dpl200</i> <i>his1Δ::dpl200/his1Δ::dpl200</i> <i>URA3/ura3Δ::imm⁴³⁴</i> <i>IRO1/iro1Δ::imm⁴³⁴</i>	This study
<i>19.7091Δ/19.7091Δ</i>	<i>19.7091Δ::C.m.LEU2/19.7091Δ::C.d.</i> <i>ARG4</i> <i>arg4Δ::dpl200/arg4Δ::dpl200</i> <i>leu2Δ::dpl200/leu2Δ::dpl200</i> <i>his1Δ::dpl200/his1Δ::dpl200</i> <i>URA3/ura3Δ::imm⁴³⁴</i> <i>IRO1/iro1Δ::imm⁴³⁴</i>	This study

<i>dap1Δ/dap1Δ</i>	<i>dap1Δ::C.m.LEU2/dap1Δ::C.d.ARG4</i> <i>arg4Δ::dpl200/arg4Δ::dpl200</i> <i>leu2Δ::dpl200/leu2Δ::dpl200</i> <i>his1Δ::dpl200/his1Δ::dpl200</i> <i>URA3/ura3Δ::imm⁴³⁴</i> <i>IRO1/iro1Δ::imm⁴³⁴</i>	This study
BY4742 <i>S. cerevisiae</i>	<i>MATαhis3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>	(Baker Brachmann et al., 1998)
R21 <i>Aspergillus</i> <i>nidulans</i>	(<i>paba A1 yA2</i>)	(Fantes and Roberts, 1973)

Appendix B: Primer sequences

Primer Name	Primer Sequence
qPCR primers	
<i>YHB1</i>	AGATCTGCTGAAACTAGAGC-3'
<i>YHB1</i>	TACACATCATGTTCTGGAGC
<i>ACT1</i>	GCCCAATCCAAAAGAGGTAT
<i>ACT1</i>	AGCTTCGGTCAACAAAACGT
<i>fhbA</i>	TCTCTAACATCCTCCATGAC
<i>fhbA</i>	G TTCACCAT TGATGTCATCG
<i>actA</i>	GACTCTGGTGATGGTGTTAC
<i>actA</i>	GGTAGTGGAGAAGGTGTATC
TAP tag primers	
3xFLAG forward	TTAATGGATTATAAAGATCACGATGGTGATTATAAAGATCACGA TATTGATTATAAAGATGATGATAAAGGTGGTGC NdeI and AflIII overhang
3xFLAG reverse	TAGCACCACCTTTATCATCATCTTTATAATCAATATCGTGATCTTT ATAATCACCATCGTGATCTTTATAATCCA NdeI and AflIII overhang
TEV-ProtA	CAAcgtacgCTACTGCTAGCGAGAATTG BsiWI cut site in lowercase
TEV-ProtA	CAActtaagTTAATTCGCTCTACTTTTCG AflIII cut site in lowercase
Spacer forward	TACGAGGTCGACTCCGGTTCTGCTGCTAGTGG NdeI and BsiWI overhang
Spacer reverse	GTACCCACTAGCAGCAGAACCGGAGTCGACCTCG NdeI and BsiWI overhang

ChIP primers

NORE up	TAGGTTAAAGCGCTCCGGCATTTC
NORE down	GTTTAATGGTTTTCCCGCTGATGCTCC
2.5 kb downstream of NORE up	AGAACCAAATCCAAAGCCTGTTGACACC
2.5 kb downstream of NORE down	AGTGCATGTAATTTTCGATATCAGTGAGC

Deletion primers**Universal Primers**

<i>C. maltosa</i> <i>LEU2</i> up	AGAATTCCCAACTTTGTCTG
<i>C. maltosa</i> <i>LEU2</i> down	AAACTTTGAACCCGGCTGCG
<i>C. dubliniensis</i> <i>ARG4</i> up	GATTCACGTTTTGAAAAAGATGTTATGG
<i>C. dubliniensis</i> <i>ARG4</i> down	GTACCAGTTAAATCTGCATCATAACATAAC
Universal primer 2 For <i>ARG4</i> and <i>LEU2</i>	ccgctgctaggcgcgcctgacACCAGTGTGATGGATATCTGC
Universal primer 5 For <i>ARG4</i> and <i>LEU2</i>	gcagggatgcggccgctgacAGCTCGGATCCACTAGTAACG

Lowercase sequences correspond to complementary sequences (deletion primer 3/universal primer 2, and deletion primer 4/universal primer 5) for fusion PCR reaction

***orf19.5785* deletion primers**

<i>orf19.5785</i> primer 1	ACAAAGATCAAACATGCACG
<i>orf19.5785</i> primer 3	cacggcgcgcctagcagcggGAACATGGATGGATTGCACG
<i>orf19.5785</i> primer 4	gtcagcggccgcacccctgcCAGGTTATACTATACGCTGG
<i>orf19.5785</i> primer 6	CAGAAACCTGCTATACAGAC
<i>orf19.5785</i> up check	TGATGATCGGTTGTATACTC
<i>orf19.5785</i> down check	GTAGATGAATAACAAGCTGG

***orf19.7091* deletion primers**

<i>orf19.7091</i> primer 1	GGAATCTACAGGGAGTTCAG
<i>orf19.7091</i> primer 3	cacggcgcgcctagcagcggTGTAATGACTGTGTCAGTCG
<i>orf19.7091</i> primer 4	gtcagcgccgcacccctgcTCTGTATTTGTTCAACCGTG
<i>orf19.7091</i> primer 6	CTTTGTTTATGTGTCTGCTG
<i>orf19.7091</i> up check	AGACTGTATTTGCTCCATG
<i>orf19.7091</i> down check	CAGTCGTATTGGCTGACAAC

DAPI deletion primers

<i>DAPI</i> primer 1	AATTATCTCGTCGTTGCCTG
<i>DAPI</i> primer 3	cacggcgcgcctagcagcggGACTGTAGTTTCTTTTAGTTG
<i>DAPI</i> primer 4	gtcagcgccgcacccctgcGTATTGACTAGTATAGAACG
<i>DAPI</i> primer 6	CATAATGGAGACATTGGTACACG
<i>DAPI</i> up check	AAACGAATAATGTTGAGGAG
<i>DAPI</i> down check	AGTGTATTATTTTCAGCAACCTC

9xMyc 6xHA primers

Cta4 _{9xMyc}	5' - ATAGTGACGATCCCAATGCTTGTCTGTCTCG
Cta4 _{9xMyc}	5' - AAAGTGAAGACTTGGCCAAATCATGTTTGG
Cta4 _{6HA}	5' - CAAggtaccTAGAGCCAATGGGAAATAATCCCGAT Acc65I cut site in lowercase
Cta4 _{6HA}	5' - CAActgacgCCATAAAAATATCCATCATCCATAAGG BsiWI cut site in lowercase
Cta4 _{6HA}	CAAgcgccgcGTTGATGTCCCTTTTTTGC NotI cut site in lowercase
Cta4 _{6HA}	5' - CAActcgagACAAGCAGATAAAGAAATTAAGAGATG XhoI cut site in lowercase

***S. cerevisiae* primers**

<i>ScYHB1</i> promoter up	CAAGaattcTTTGTAACCCTCATGCTCG EcoRI cut site in lowercase
<i>ScYHB1</i> promoter down	CAAtgatcaTCGGCTAGCATAATGAATAAAGTCTTTG BclI cut site in lowercase
9XMYC Tag	TAAcacgtcAAATGCAATTTGG BmgBI cut site in lowercase
9XMYC Tag	CAActgcagTTTAGCTAGTGGATCCGTTCAAG PstI cut site in lowercase

Appendix C: Codon optimized sequence of *CTA4_{9xMyc}* for expression in *S. cerevisiae*

ATGACATCTGAACATAAAGTATCAAGAAACAAACCTTCCTTGTCTTGTACAA
 ACTGTCGGAAACGTAAGACCAAATGTGATAGAAAACAACCATGCTCCGCTTG
 TGTAAGAGAAAATTAATTTATCTTACACTTGTGCTTATGACACTCCACCAAAAC
 AATTTGCAATTAATTCTGATACTCCCCAACGGAATATCCAGCACGCAAGCGT
 CACAAACCTGAAACCATTTGATGATGCTGAGAACCCAGTTCAACAAGAATTGG
 CTAGATTGCGGAAACGGATTATTGAGTTGGAAACTTCATTACAGCATGCCCA
 CACACCAAAAACACCTCCGGAAAACAAAACTATCCGAGAAACAAACCCAT
 ATTATCACCAACAAGCTCTACTTGTCTAATCCTATTGTGAATGACGAAGAGA
 TTATCAACTTTTACGAAGGCTATACCCCAATTCAAGTTAAACGTAATATTAGA
 AGAGTCAATTATGGGCCATTGTCATGGATTGCCTTGATGAGAAAAGATCCAG
 CATTAAACTTGGCTTGGAAAGTGTTATGTAGCAAGGATTCCTATTGTATAATT
 ACCAATCGATGGCCCCGTTTGACTTCAGAAAACAATGCCTTAATCAATATTTTC
 CCACAACCCCGAGGATGAGAAGAATTCCAACCTACATGGACAAAATATTTTCAG
 AAAAGAGTGTTAGAAATTTGAAGGTTATGACGAGTTTGTTCCTTATAGATCTCT
 TTATGGTCGAAAGGAGTCAGATACCACCAACACAGAACAACCTACAACATCC
 GACAAATCCACATCAAAACCTTCCTCAACAACAACCTACTAATCCTGGACCAC
 GGTAAATTTTCACTCAAATCAGTCTTGCTAAATCTATATTTGATGGTCGTATA
 AACCCCGAGCTACATTTGATTGAGAAAATTAACAAATCGTACCTAAAAGAA
 AAGTTTTTTTGGACATTAATGGATCGATTTTTTCACAGATATATACCCTTATTTCC
 CATTTGTGGATGAAGAATCATTCAAAAAAGAGCTTTTGAGAATATTTGGTAC
 GAGAACTTACACTGATTTACCCTTTGAAAGTATTAGGATCGAGAAAAAGTTA
 GATTTGGCCAATATAGCAATTTGTTTTATTTTGCTACGGTTAACATATTTGTCA
 TTTTTCAATAACCGTAATTGTGTCAATGAATTTATTATGAATTCTGAAGAAGA
 AACTGTGGCAAAATACATTATGCAAAATCCTATCAATTTATCTACTATAGAAG
 TGGCAAATTCATGTATTCAATGTTTCCAATTCACAAGAAAATCCAATATAACT
 GTTTTCCAAGCAATGTTGTATATGAGAATCTATCGAAATTGTGCACCTGAAGA
 AAGCGATGGAATTGATGGTGGTGACTCTCAGGTTGCTACTGCACTACTAGTG
 CAAATGGCGTATTCATTAGGACTTAATCGTGAACCAGATAAATTTGATGTCTG
 CAACGATGAAAAAGTTAACCATTTAGGACGGAAAATGTGGAGTTTTTTAATG

AGATCAGATTTAACTCAATGCTACACTTTGGGTAATCCAACAAGTATAGATCC
GAGAAATTATGATGTTAGACCTCCATTTGTAAATGACAATAACTCAAATATTG
AAGATAGAGAAATGGAAATTGCTATTGTTGAAACATTTGTCTATTTGAATTCA
GAGTTGGATCTAATGCGTAAAATGCTAGATCACGTGCTAGATATCAATAATG
GTGCCAAAATTAATGATTTGACTAGAGATGTACATGACTTTGAAGTGATTTTG
CATGATTTCTTCAATGTTTTAGGAAACGTTAATGCCGGTAAAGTGAAAACCTGA
TAGAGAAAGCGTGATTCAATATTACCATGTTATTAAATTGAAGATTTTTTTGT
CGATGAAAACAGCATTGGTTTCACTTTATTTCCACATTTATTTGCATTATGAG
AAGAGATTTGATAGCGAATTGTCATTTTTCTATTTGTCAAAAATGTACCTAAT
AATAAGAGATGATTTGATACCTGCTATTGGTCACACATTGATAGGTAAATAC
AGTTCCAAGGCTTTAGTTTTGAATCCTAACATGCAATTGGCTTTACACAAAAT
CAATCAAATTAAC TTGTCGTGTTTAATTCGTGTTAGTTATTTAGTGCACATGTT
TGAGCAAAAACCAAATCATGAAGAATTGATGGCAAATGATATAGTGTATTTG
GAACATTTCAATAAACTCAAGAGCTTGATGACAAATGTTAGACGTACTGGTG
ACTTTGTTAGGGTTATTATGGAACGAATTGGATCTAGATATTACTATTCATGG
CGTATAGCGAAAGCTCACGTTGGATTATTCAAAATGTTATCACAACTGAAA
ATTACGATAAGCAATTGTCTGGGATAAGGAAATTAAAGTCTTTCCAATTCACT
CCAAAGCAATTGGATGAACTAGACAATTTGATCCGTATTTTAGGGAAATGTG
CTGAGAAGTTAGTCTTTTATGAAGATAGTGTTGGTATGTTGCCCGATTTTGAA
ATGCCATTCAAACCCTCTGAAGGTGATGGAAATGAAAAAGATCAAAATCAAA
ATGACAGCAAAAACCACCACCCTACTACTACTACTACTACTTCCGATTCAAC
AGGTATATCAATAACACCCGAGAATAATGCATCGGCAATAGTGACACCTTAT
TCATCACTTGCATCCCCTCGACTCCACTTGGACAATATTAGCAATGATTACGT
TGATCAAATGTGGTTTTTTGATGATGGCAATGAAATATGATACTCTAGAGCCA
ATGGGAAATAATCCCGATTGCCAAAACCTGTAATAGTGACGATCCCAATGCTT
GTCCTGTCTCGTATAATACTCGTGACAATGGAGTCTTAGGTGGTACTGGTGGT
CAACTACTTACACAACAAAAACAAATGACGGCACAGGAACAATTAGCGCAA
CAAGAGATAACACGTCAAATGCAATTTGGTAACGAGAATGGATACAACAATG
GATTCTTTCTTAATTCTGGATTTGACCTTATGGATGATGGATATTTTTATGCAT
ATGAGGTCGACTCCGGTTCTGCTGCTAGTGGTGAACAAAAGTTGATTTCTGAA
GAAGATTTGAACGGTGAACAAAAGCTAATCTCCGAGGAAGACTTGAACGGTG

AACAAAAATTAATCTCAGAAGAAGACTTGAACGGATCCTCTAGAGGCGAACA
AAAGTTGATTTCTGAAGAAGATTTGAACGGTGAACAAAAGCTAATCTCCGAG
GAAGACTTGAACGGTGAACAAAAATTAATCTCAGAAGAAGACTTGAACGGA
TCCTCTAGAGGTGAACAAAAGTTGATTTCTGAAGAAGATTTGAACGGTGAAC
AAAAGCTAATCTCCGAGGAAGACTTGAACGGTGAACAAAAATTAATCTCAGA
AGAAGACTTGAACGGATCCACTAGCTAA

Appendix D: Genome-wide expression profile of *C. albicans* in response to nitric oxide, nitrite, nitrate or thiocyanate

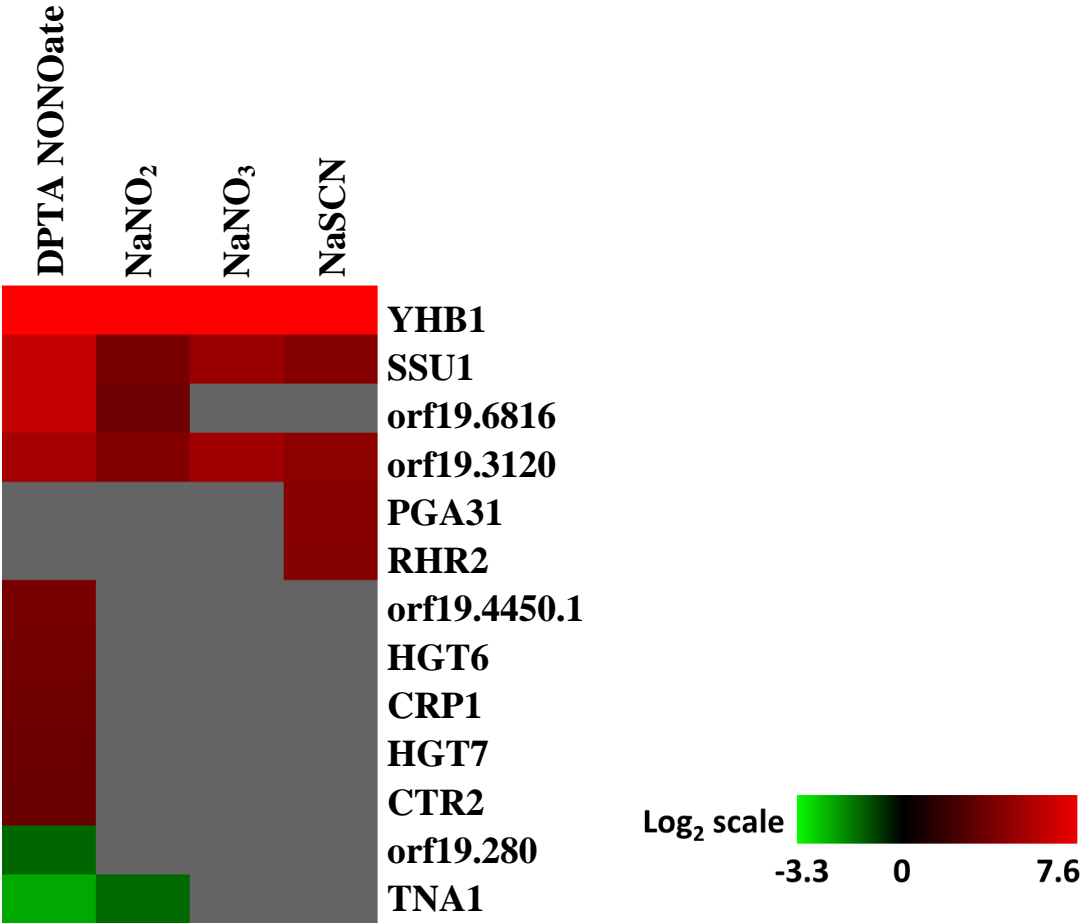


Figure S1. Genome-wide expression profile of *C. albicans* in response to nitric oxide, nitrite, nitrate or thiocyanate

Log-phase wild-type *C. albicans* cells (strain SN152) grown in rich yeast media, pH 6, were treated for 10 minutes with 5 mM NaNO₂, 5 mM NaNO₃, 5 mM, 0.02 mM DPTA NONOate or 5 mM NaCl (control). All expression values, from averages of two biological and technical replicates, are represented as the ratio of expression levels in treated versus control sample. Ratio values were converted to log base 2 and visualized using Treeview (Eisen et al., 1998). In this “heat map” red indicates induction and green indicates repression. Only genes with *P* values <0.05 and 2 fold or greater change in expression are shown. Grey boxes indicate either the expression values were not statistically significant or there was less than a 2 fold change in expression.

Appendix E: Growth inhibition of *C. albicans* by nitrite with or without nitrate or thiocyanate pretreatment

Table 2. Growth inhibition of *C. albicans* strain SN152 (wild-type), *cta4Δ/Δ* and *yhb1Δ/Δ* by nitrite with or without nitrate pretreatment in YEPD, pH 6.05

Nitrite conc. mM	OD ₆₀₀ ± 1 S.E.M.					
	SN152		<i>cta4Δ/Δ</i>		<i>yhb1Δ/Δ</i>	
	No <u>pretreat</u>	<u>pretreat</u>	No <u>pretreat</u>	<u>pretreat</u>	No <u>pretreat</u>	<u>pretreat</u>
0	0.74 ± 0.008	0.74 ± 0.008	0.76 ± 0.005	0.75 ± 0.006	0.75 ± 0.005	0.75 ± 0.006
5	0.73 ± 0.006	0.73 ± 0.005	0.73 ± 0.004	0.73 ± 0.004	0.67 ± 0.006	0.68 ± 0.004
10	0.66 ± 0.010	0.73 ± 0.007	0.63 ± 0.005	0.65 ± 0.003	0.43 ± 0.007	0.45 ± 0.004
15	0.56 ± 0.016	0.72 ± 0.010	0.43 ± 0.034	0.47 ± 0.021	0.32 ± 0.007	0.35 ± 0.008
20	0.19 ± 0.011	0.70 ± 0.008	0.14 ± 0.004	0.15 ± 0.003	0.16 ± 0.003	0.19 ± 0.007
25	0.15 ± 0.001	0.16 ± 0.007	0.13 ± 0.004	0.13 ± 0.004	0.14 ± 0.003	0.14 ± 0.002
30	0.13 ± 0.002	0.14 ± 0.002				

Table 3. Growth inhibition of *C. albicans* strain SN152 (wild-type) by nitrite with nitrate or thiocyanate pretreatment in YEPD, pH 3.5

Nitrite conc. μM	$\text{OD}_{600} \pm 1 \text{ S.E.M.}$		
	<u>No pretreatment</u>	<u>1 mM Nitrate</u>	<u>1 mM Thiocyanate</u>
0	0.88 ± 0.004	0.90 ± 0.002	0.88 ± 0.005
100	0.60 ± 0.007	0.72 ± 0.006	0.80 ± 0.008
200	0.44 ± 0.005	0.54 ± 0.007	0.69 ± 0.006
300	0.22 ± 0.007	0.35 ± 0.004	0.60 ± 0.006
400	0.18 ± 0.014	0.27 ± 0.013	0.54 ± 0.010
500	0.13 ± 0.007	0.18 ± 0.006	0.48 ± 0.008
600	0.10 ± 0.002	0.13 ± 0.008	0.41 ± 0.005
700	0.10 ± 0.002	0.10 ± 0.002	0.35 ± 0.005